How do gut bacteria boost our immune system?

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ABSTRACT

It is well known that the gut microbiota plays a role in numerous systems in our body through multiple mechanisms and that alterations in its functional composition, can lead to disease. Large-scale research programs have allowed the identification of new strains and/or new microbial functions and components supporting the development of potential therapeutic applications. One approach is the use of live biotherapeutics. *Lactobacillus* species are one of the most widely used live biotherapeutic products and can be found in a large variety of food products throughout the world. *Limosilactobacillus reuteri* (*L. reuteri*) is an excellent model organism to identify host-specific immunomodulatory properties of commensal bacteria because it is widespread gut symbiont found in many vertebrate hosts and one of the first to colonise the human gut. The underlying mechanisms by which *L. reuteri* exerts its well-documented host-specific therapeutic effects in the gut are not fully characterised. *L. reuteri* strains express cell-surface adhesins such as mucus binding protein (MUB) or serine rich repeats proteins (SRRP) which mediate bacterial adhesion to the host tissue. These adhesins has recently been shown to be glycosylated and can be recognised by lectins. This project will test the hypothesis that the ability of *L. reuteri* strains to colonise the gut and trigger immune response is mediated by protein-glycan interactions occurring between cell-surface structures and lectins found at the mucosal interface and immune cells.

We showed that the binding of the human (PTA ATCC 6475) and pig isolates (ATCC 53608) to mucin and epithelial cells was strain-dependent and that the binding was shown to be dependent on the surface expression of CmbA and MUB. Moreover, using *ex-vivo* binding assay, we demonstrated both the expression and glycosylation of SRRP is important for the adherence of the rat isolate (100-23 strain) to the mice forestomach. Here, we demonstrated that host strain-specific glycosylated adhesins $SRRP_{100\text{-}23}$ and MUB_{53608} contribute to the immunomodulatory effects of *L. reuteri* 100-23 and ATCC 53608 *in vitro* by (i) mediating enhanced surface activation marker expression and (ii) inducing pro-inflammatory and anti-inflammatory cytokines by DCs. Using BMDCs lacking surface expression of Dectin-2, we also showed that Dectin-2 contributed to the *L. reuteri* 100-23–induced production of cytokines and internalisation by DCs. Moreover, we purified and characterised the *L. reuteri*-derived bacterial extracellular vesicles (BEVs) and showed that they could mediate bacteria-host interactions.

The data produced in this work provided novel insights into how *L. reuteri* cell surface glycosylation plays a crucial role in the interaction with the host's immune system furthering our understanding of the underpinning mechanisms behind their beneficial interactions with the host.

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ABBREVIATIONS

Table 1 Abbreviations

ABC	ATP-binding cassette
AFM	Atomic force microscopy
APC	Antigen-presenting cell
aSec	Accessory secretion system
Asp	Accessory sec proteins
BEVs	Bacterial membrane vesicles
BMDCs	Bone marrow derived DCs
BR	Binding regions
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFDA	Carboxyfluorescein diacetate
CFU	Colony forming unit
CHAPS	(3-[(3cholamidopropyl) dimethylammonio]1-propanesulfonate)
CmbA	Cell and mucus-binding protein A
ConA	Concanavalin A
CRD	Carbohydrate recognition domain
CTLs	C-type lectins
CTV	Cell trace violet
DAPI	4’,6-diamidino-2-phenylindole
DCs	Dendritic cells
DC-SIGN	DC-specific ICAM-3–grabbing nonintegrin
Dectin-1	Dendritic cell-associated C-type lectin 1
Dectin-2	Dendritic cell-associated C-type lectin 2
DMBT1	Deleted malignant brain tumours 1
DSS	Dextran sodium sulfate
E. coli	*Escherichia coli*
EA	Epithelium-associated
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol-bis (b-aminoethyl ether)-N, N, N', N'- tetraacetic acid
EF-Tu	Elongation factor T
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
EPS	Exopolysaccharides
FBS	Fetal bovine serum
FcRγ	Fc receptor γ chain
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
FSC	Forward scatter
FTF	Fructosyltransferase
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography – mass spectrometry
GF	Germ-free
GI	Gastrointestinal
GlcNAc	N-Acetylglucosamine
Glu	Glucose
GM-CSF	Granulocyte macrophage colony stimulating factor
GMBS	G -maleimidobutyryl-oxysuccinimide ester
Gram-	Gram negative
Gram+	Gram positive
GSL-1 B4	Griffonia simplicifolia Lectin I isolectin B4
Gtf	Glucosyltransferase
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HePS	Heteropolysaccharides
Hex	Hexose
h-INTL1	Human intelectin-1
HIP/PAP	hepatocarcinoma-intestine-pancreas/pancreatic-associated protein

HoPS	Homopolysaccharides
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IECs	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IL	Interleukin
IMAC	Immobilised metal affinity chromatography
IRIDA	Integrated Rapid Infectious Disease Analysis
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
kDa	Kilodalton
L. reuteri	*Limosilactobacillus reuteri*
LDM II	*Limosilactobacillus* defined medium II
LPS	Lipopolysaccharides
LSP	Large surface protein
MALDI-ToF	Matrix assisted laser desorption/ionisation – time of flight
MAMP	Microbial associated molecular pattern
MHC	Histocompatibility complex
moDCs	Human monocyte derived DCs
MOI	Multiplicity of infection
MOPs	3-(N-morpholino) propanesulphonic acid
MRS	De Man, Rogosa and Sharpe
MTS	3-mercaptopropyltrimethoxy silane
MUBs	Mucus binding proteins
MyD88	Myeloid differentiation primary response 88
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear factor-κB
NKC	Natural killer-gene complex
NLRs	Nucleotide-binding oligomerization domain-like receptor
NMR	Nuclear Magnetic Resonance
OD_{600nm}	Optical density at 600 nm
OMVs	Outer membrane vesicles

PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PBST	PBS, supplemented with 0.1% Tween®20
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
pIgR	Polymeric Immunoglobulin Receptor
pIVOC	Polarised *In Vitro* Organ Culture
pPGM	Purified porcine mucin
PREDICT	Personalised Responses to Dietary Composition Trial
PRR	Pattern recognition receptors
qPCR	Quantitative PCR
RBC	Red blood cell
RCA	Ricinus communis agglutinn
REG3	Regenerating islet-derived protein 3
Rha	Rhamnose
RIG-1	Retinoic acid-inducible gene I
RT	Room temperature
ROS	Reactive oxygen species
SCFAs	Short acid fatty acids
SecA2	Accessory secretion system protein A
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SIF	Simulated intestinal fluid
SIG	Simulated gastric juice
SIgA	Secreted immunoglobulin A
sPLA2	Secretory phospholipase A2
SRR	Serine-repeat-rich
SRRPs	Serine rich repeat proteins
Syk	Spleen tyrosine kinase
TEDs	Trans-epithelial dendrites
TEM	Transmission electron microscopy

TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor beta
TH	T helper cell
TLR	Toll-like receptor
TMB	3,3',5,5'- Tetramethylbenzidine
tMUB	Truncated MUB
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UDP	Uridine diphosphate
WB	Western blot
WGA	Wheat germ agglutinin
WT	Wild type

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INTRODUCTION

1. THE GASTROINTESTINAL TRACT

1.1 Anatomy and physiology of the GI tract

The gastrointestinal (GI) tract is a muscular organ that stretches from the oral cavity to the rectum[1]. The human GI tract is divided into different anatomical chambers and their structural arrangements vary to fit their specific functions[2, 3]. The upper region is comprised of the mouth, pharynx, oesophagus, stomach and the duodenum, while the lower region consists of the small and large intestines. The human small intestine, which it is approximately 6 meters in length, is subdivided into the duodenum, jejunum, and ileum **(Figure 1A)**, providing a large surface area for absorption of essential nutrients[3, 4]. In the duodenum, digestive enzymes secreted by the pancreas and the liver breakdown proteins and bile emulsifies fats into micelles[5]. The duodenum also contains Brunner's glands which release alkaline fluids that neutralise acid and prevent acid chyme of the stomach[6]. The start of the jejunum is marked by a sharp bend, the duodenojejunal flexure. The jejunum is where most of the food digestion and absorption occurs. The final portion, the ileum, is the longest segment of the small intestine which is thicker and more vascular than the jejunum[3, 5] (**Figure 1A**).

The human large intestine is a 1.5 m long, 7.5 cm wide, horse-shoe shaped tube, which consists of several distinct regions: the caecum, ascending, transverse, descending, sigmoid colon and rectum[7] (**Figure 1A**). The caecum is an expanded sac that receives semi-digested food from the ileum and starts to concentrate waste material into faecal matter. The colon does not play a major role in nutrient absorption[8]. Instead, it stores waste like indigestible fibre, absorption of fluids and electrolytes, and certain fat-soluble vitamins (such as vitamin k, vitamin B12, riboflavin, and thiamin)[9]. The wall of the colon is made up of several pouches (haustra) that are constantly under tension by three thick bands of muscle (taenia coli). The rectum is the final 15 cm of the large intestine. It expands to hold faecal matter before it passes through the anorectal canal to the anus. Thick bands of muscle, known as sphincters, control the passage of faeces.

Furthermore, the GI tract hosts trillions of resident bacteria (referred to as the 'gut microbiota'), a network of intestinal immune cells and a complex enteric neural network (often refers to the body 'second brain'). The gut microbiota plays a preponderant role in the regulation of metabolic, endocrine and immune functions[10] (**see section 2**).

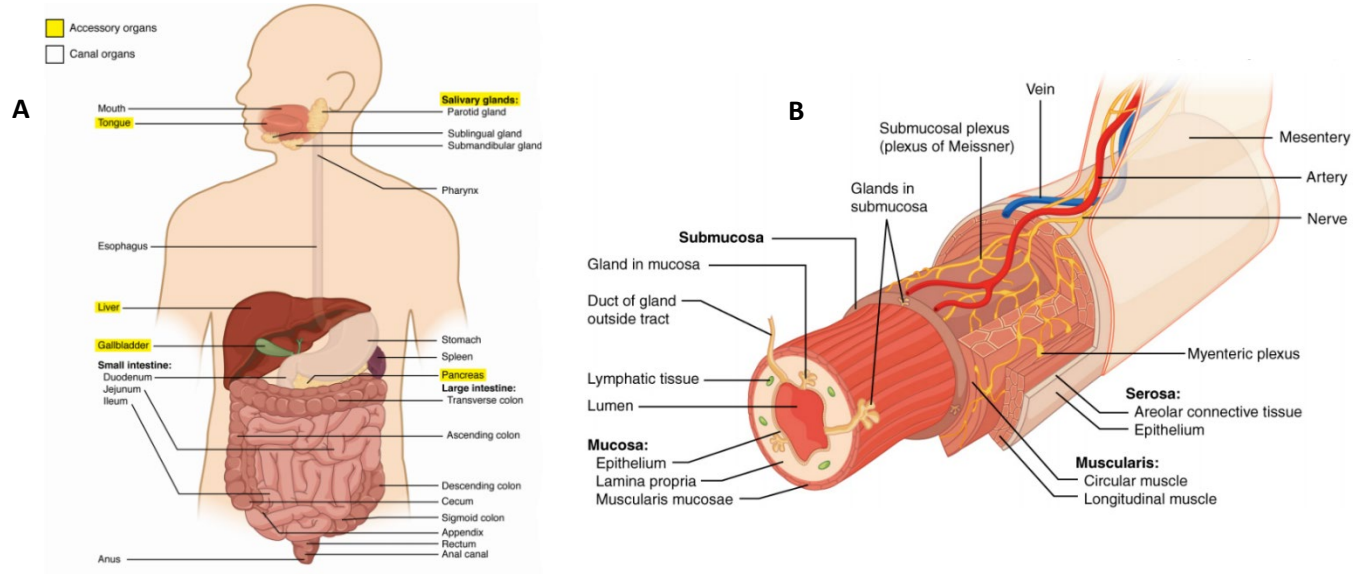

Figure 1: Overview of the human gut.

(A) Components of the GI tract. All digestive organs play integral roles in the life-sustaining process of digestion. **(B)** The wall of the GI tract has four basic tissue layers: the mucosa, submucosa, muscularis, and serosa. Taken from Biga et al., 2019.

Throughout its length, the wall of the human GI tract is organised into four specialised layers: the mucosa, submucosa, muscularis externa, and adventitia or serosa[11,4] **(Figure 1B)**. The mucosa comprises an epithelial lining, including the glandular tissue, a lamina propria (LP) of loose connective tissue, which provides vascular support for the epithelium[12-16] and the muscularis mucosae, which is a layer or two of smooth muscle fibres, separating the mucosa from the submucosa[15]. Surrounding the mucosa is the submucosa, which is made up of blood vessels, lymphatics, various connective tissues, to allow nutrient transfer away from the gut[17]. Large arterioles, venules, and lymphatic vessels enable numerous penetrating capillary vessels to supply and drain most of the mucosa and muscularis externa[17]. The connective tissues support the mucosa and connect it to the muscularis layer. The muscularis propria is formed of thick bundles of smooth muscle fibres, which are arranged as two distinct sublayers: an inner layer of circular muscle that runs in a circular fashion; and an outer layer of longitudinal muscle that runs in an up and down fashion[11]. Between the two muscle sublayers sits the myenteric nerve plexus which enables the smooth muscles to produce rhythmical waves of contraction and relaxation known as peristalsis which moves food throughout the gut. The final layer of the gut, the serosa, is a thin layer of the GI canal superficial to the muscularis. This layer is mostly composed of a layer of mesothelium and the underlying connective tissue, blood vessels and lymphatic tissues and visceral peritoneum.

Murine models have been widely used in biomedical research[18]. Extensive similarities in anatomy, physiology and genetics have allowed numerous inferences about human biology to be drawn from murine experimentation **(Figure 2)**. However, the anatomy of the mouse and human GI tract also have prominent differences, which might be shaped by their diverging diets, feeding patterns, body sizes and metabolic requirements. In addition, even though the average ratio of intestinal surface area:body surface area is similar between mice and humans[19], this ratio differs greatly between the two species over different sections of the gut. For example, the average small intestine:colon length ratio is 2.5 in mice versus 7 in humans[20], and the surface ratio of small intestine:colon is only 18 in mice compared to 400 in humans[19]. The mouse cecum is also large relative to its GI tract and is an important site for the fermentation of plant materials as well as for the production of vitamin K and B, which mice reabsorb through coprophagy[19]. By contrast, the human cecum is relatively small, with an anatomical structure similar to that of the colon[18]. Further, the gut microbiota composition is known to differ between mice and humans[21]. The most discernible difference is the ratio of the two major phyla, with humans having a greater Firmicutes:Bacteroidetes ratio, whereas the inverse is true for mice[22-26]. These differences should be considered during experimental design and interpretation.

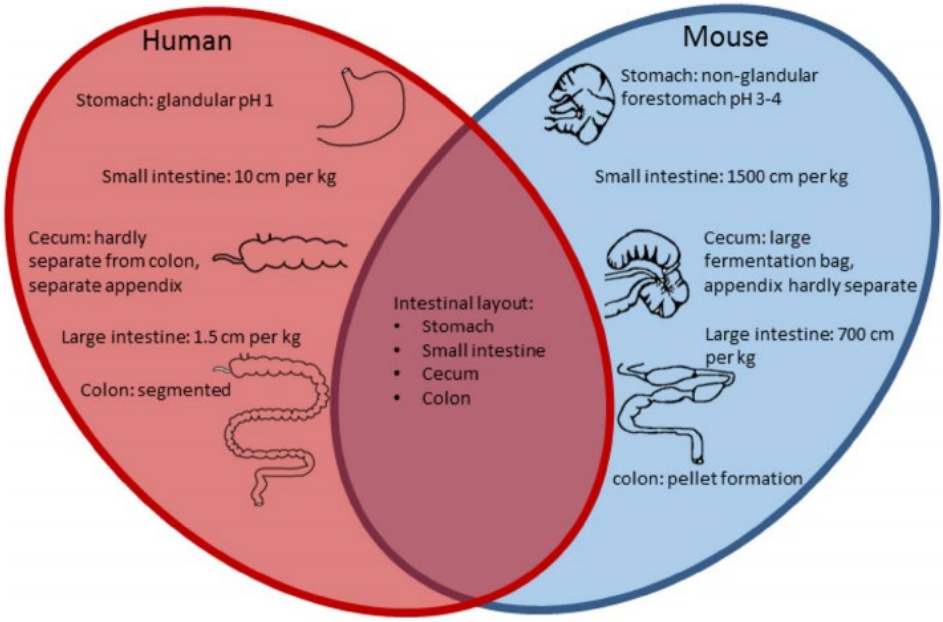

Figure 2: Comparison of the intestinal tract features of human and mouse.

The human colon is divided into different sections with the presence of taenia coli and compartmentalisation in haustra, which are absent in the mouse colon. The human stomach is lined with a glandular mucosa that secretes gastric acid, whereas the mouse stomach is divided in two regions – a glandular stomach and a non-glandular or fore-stomach. The mouse glandular stomach is responsible for secreting gastric acid, whereas the non-glandular stomach functions as a temporary site of food storage and digestion. Taken from Hugenholtz and M. de Vos, 2018

One of the most anatomical remarkable differences between these two species, is the presence of a non-glandular forestomach in mice that is absent in humans. The murine forestomach is lined with keratinizing squamous mucosa and covers two-thirds of the entire stomach. The forestomach has no secretory activity and is used for food storage[27] and is covered with a biofilm comprised of strains of various *Lactobacillus* species.[28, 29]. Although *L. reuteri* and *Lactobacillus johnsonii* are found throughout the mouse GI tract, there is a strong indication that the forestomach is their main habitat and that the caecal populations are composed of cells that have descended from the forestomach populations[30]. Comparative genomic analysis has shown that the murine *L. reuteri* strains are very different from those found in humans and have urease genes to cope with low pH and a variety of rodent-specific genes which, when inactivated, affects their persistence in mice[31].

1.2 The intestinal mucosa

The intestinal mucosal barrier, also referred to as intestinal barrier, is a heterogeneous entity composed of microbial, biochemical and immune elements produced by the intestinal mucosa **(Figure 2)**[32, 33]. The central components of the intestinal barrier are the mucosal and epithelial layers, which provide a physical separation between the lumen and the body[32]. Intestinal epithelia form a selective barrier that halts passage of commensal bacteria and pathogens while allowing intercellular flux of molecules and ions simultaneously[34].

The secretion of various molecules into the lumen also reinforces the barrier function on the luminal side, while a variety of immune cells provide additional protection below the epithelial layer[35]. In the intestines, these include antimicrobial peptides and mucins, which are predominantly synthesised by Paneth and goblet cells, respectively. Secreted mucins form a protective mucus layer that covers the apical surface and limits direct interactions of the epithelium with microbes and larger molecules, such as food particles[36, 37]

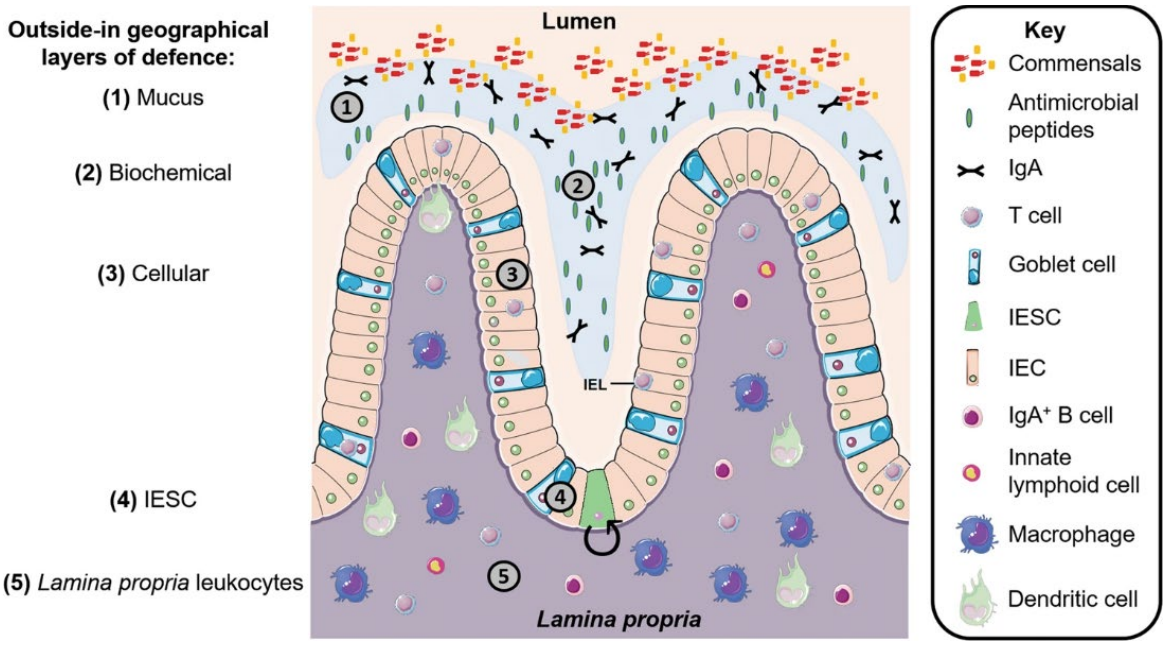

Figure 3: Components of the intestinal (colon) barrier.

Several immune effectors function together to segregate luminal microbes and to minimize bacterial-epithelial contact. These include **(1)** the mucus layer, which acts as a physical barrier **(2)** that is further reinforced biochemically with epithelial antibacterial proteins, and immunoglobulin A (IgA) secreted by LP plasma cells. **(3)** Intestinal epithelial cells form a single-cell layer of protection which is interspersed with intraepithelial lymphocytes. **(4)** Within intestinal crypts are intestinal epithelial stem cells, which are key in replenishing the epithelial surface. **(5)** Beyond the epithelial layer is the LP, which is densely populated with leukocytes that serve to back up the innate immune defences and provide immunological memory against future repeated insults. Taken from Thoo et. al., 2019.

1.2.1 Intestinal mucus organisation

The luminal side of the GI tract is lined by viscoelastic adherent mucus gel layers secreted by intestinal goblet cells and submucosal glands in the epithelium[37, 38]. This highly dynamic matrix plays a key role in gut homeostasis; particularly, serving as a protective barrier against chemical and enzymatic attacks of the epithelium as well as shielding from microbes[39-41]. Intestinal mucus is primarily composed of water (approximately 95%) and branched glycoproteins (including mucins) that interact with the external environment and through their hydrophilic properties, determine mucus viscosity[42, 43]. In addition to secreted mucins, the mucus layer is comprised of water, lipids, carbohydrates, and molecules of the immune systems such as anti-microbial peptides and lectins which facilitate the clearance of pathogenic microbes[44, 45], and secreted immunoglobulin A (SIgA)[46]. Although mucus located throughout the gut contains the same biological components, the organisation and thickness of this layer varies along the GI tract[47] **(Figure 4)**.

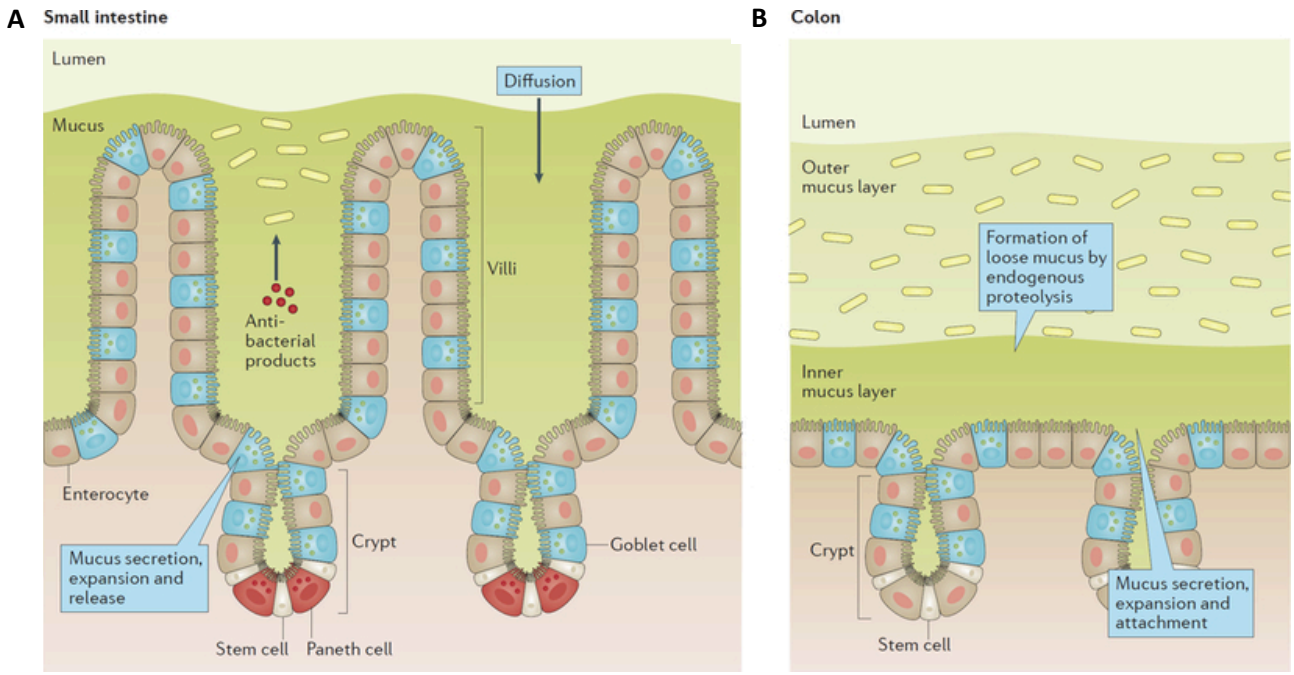

Figure 4: The structure of the mucus layer varies with regional locations within the GI tract.

(A) The small intestine contains a single layer of mucus, which is loosely attached to the epithelium and easily penetrable. Bacteria within the small intestine are primarily repelled from the epithelium by antibacterial modulators. **(B)** The distal colon contains two mucus layers; a stratified adherent inner mucus layer and loosely adhesive outer mucus layer. The inner mucus layer of the colon is essentially sterile and the outer mucus layer harbours the intestinal microbiota. Taken from Johansson and Hansson, 2016.

The total mucus thickness is estimated in mice to be approximately 500 µm in the duodenum, 250 µm in the jejunum and 200 µm in the ileum, whereas in rats it is approximately 170 µm in the duodenum, 124 µm in the jejunum and 480 µm in the ileum [40]. The stomach and colon have mucus bilayers[48]. The stomach comprises an inner compact mucus layer and an outer loose mucus layer which contains MUC5AC mucin produced by the epithelium[40, 49, 50]. In contrast, the small intestine mucus is made of a single discontinuous and less well defined layer , allowing the passage of nutrients and the release of digestive enzymes localised in the brush border membrane of epithelial cells[51] (**Figure 4B**). The small intestinal mucus in mice is penetrable to beads equivalent to the size of bacteria (i.e., 0.5–2 µm)[52]. In the small intestine, mucus also plays role in host immunity by delivering tolerogenic signals through interaction of mucin glycans with host lectins[53]. Recently it was shown that goblet cells can form goblet cell associated passages (GAPs) and deliver luminal substances to underlying LP antigen-presenting cells (APCs) in a manner capable of inducing adaptive immune responses (see[54] for a review).

The distal colon contains a loose outer mucus layer which is a habitat for the gut microbiota, and a stratified inner mucus layer which prevents these bacteria to contact the epithelium[16] (**Figure 4A**). Studies in mice demonstrated that the gut microbiota composition could modulate mucus barrier thickness and penetrability in ways that can have implications for health and disease[55].The causality was demonstrated using transplantation of caecal microbiota to germ-free (GF) mice, conferring mucus properties in the colon similar to that of wild-type mice[56]. Moreover, when exposed to bacterial products, the thickness of the inner mucus layer was quickly restored to levels observed in conventionally housed mice[56].

1.2.2 Intestinal mucins and glycosylation

Mucins are produced by goblet cells that are dispersed throughout the epithelium and serve as the main structural component of the intestinal mucus layer[57]. Mucins are characterised by regions rich in proline, threonine, serine amino acid residues (PTS) which are often repeated in tandem[58] and the site of heavy *O*-glycosylation[59].

Mucins are categorised into membrane-bound mucins, secretory gel-forming mucins (insoluble) and secretory non-gel forming mucins[60] (**Figure 5**). In humans, there are 21 mucins differentially expressed on mucosal surfaces[38, 40, 49, 50, 61-64]. In the small and large intestines, MUC2 (muc2 in mice) is the main gel-forming mucin whereas membrane-bound mucins can be MUC1, MUC3, MUC4, MUC13, and/or MUC17[65-67]. The five main primary oligosaccharides which decorate mucins are *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose, galactose and sialic acid[68]. Serine and threonine both

contain side chains with hydroxy groups that serve as attachment sites for GalNAc, which is added in the Golgi apparatus[68]. Each organ of the human body contains a unique repertoire of peptidyl-GalNAc transferases which extend and branch the GalNAc residues and generate a plethora of glycan epitopes with different properties and functions[69]. The sheer number of glycans added to the variability and multimeric forms of mucins makes their structural characterisation and analysis of chemical and physical properties challenging[70].

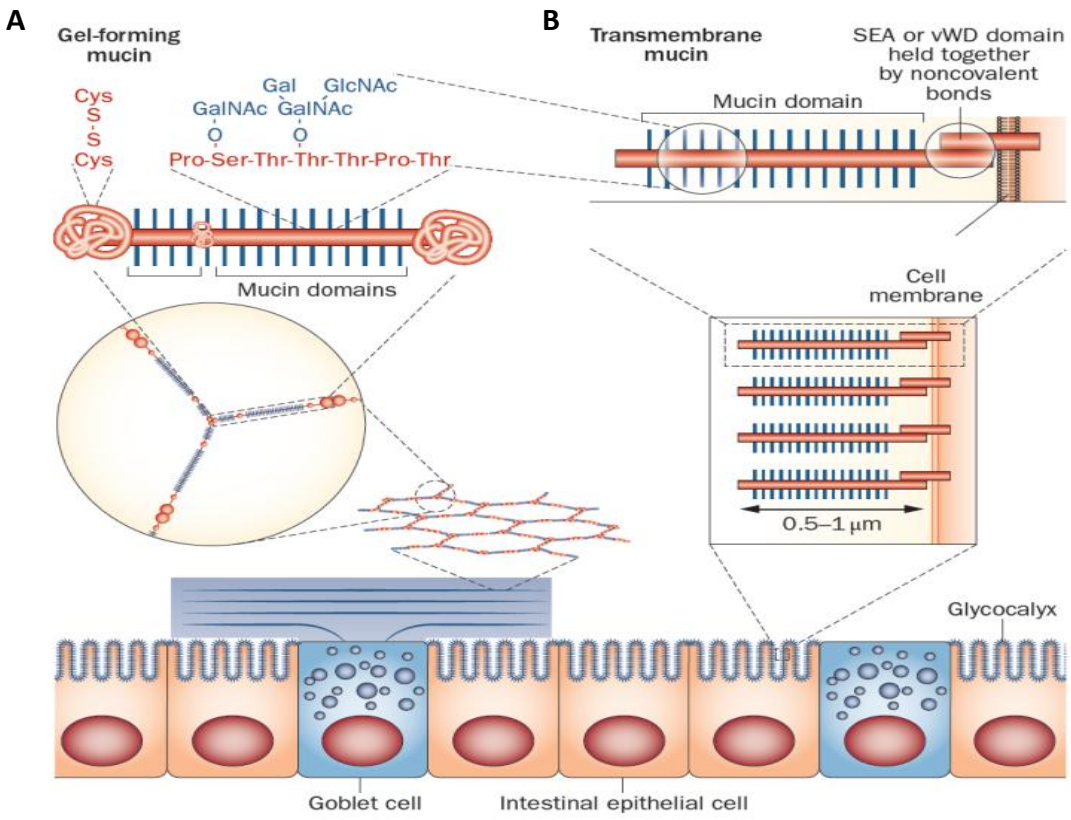

Figure 5: Model of a gel-forming mucin produced by goblet cells and transmembrane mucins attached to the apical membrane of enterocytes.

(A) the MUC2 mucin polymer is packed in the granulae (blue) of the goblet cells and during secretion expands to form flat ring-like structures that stack under each other. The enlarged ring shows the oligomeric nature of the MUC2 polymer, and above the MUC2 monomer is shown with its central mucin domains and exemplified O-glycans. The MUC2 N-termini and C-termini are held together with numerous disulphide bonds. **(B)** the apical side of the intestinal enterocytes are covered by a glycocalyx made up of mucins that are anchored in the cell membrane. The long and extended mucin domains make up the glycocalyx. Taken from Johansson et al., 2013.

Following mucus secretion, the MUC2 protein complex expands dramatically to form a net-like structure[71]. Mucin expansion occurs due to increased pH and decreased Ca^{2+} levels driven by cystic fibrosis transmembrane regulator (CFTR) channels. CFTR-mediated secretion of HCO_3^- reduces Ca^{2+} levels which weakens the ring structure of the mucin complex and allows the densely packed MUC2 mucin to expand into large flat sheets[71]. The newly secreted mucus sheets are laid down on the epithelium by interacting with previously secreted mucus and subsequently attaching to the epithelium[72]. In the colon, expansion of the outer mucus layer is also triggered by bacteria that secrete glycosidases that sequentially cleave individual monosaccharides from mucin glycans[72] to further relax the tight-knit structure of mucin glycans[73]. The turnover of the intestinal mucins is a delicate process that needs to be regulated and balanced to ensure that mucus maintains an optimal protective function[74].

Investigation the turnover of these mucins in the small intestine using *in vivo* labelling of O-glycans has shown that the turnover of Muc2 is slower in goblet cells of the crypts compared to goblet cells along the villi. Whereas, Muc17 showed stable expression over time at the plasma membrane on villi tips, in crypts and at crypt openings[75]. Chemical and bacterial hazard towards the intestinal epithelium necessitate rapid and frequent renewal of the secreted mucus layer in the villi area is combined with massive secretion of stored Muc2 from goblet cells in the upper crypt. In the colon, the intercrypt surface goblet cells continuously secrete the inner mucus layer, while the goblet cells in the upper part of the colonic crypts secrete mucus in response to stress stimuli. In mouse, the inner mucus layer in the distal colon is renewed every 1–2 hours[37].

Muc2 $^{-/-}$ mice lack a protective inner mucus layer and have direct contact of bacteria with the epithelium which has been observed spontaneously developed severe colitis and colorectal cancer[76]. The significance of mucin glycans has been demonstrated using several mouse models deficient in glycosyltransferases. Transgenic mice lacking Core-1β1,3-galactosyltransferase (C1GalT1, also called T-synthase) and Core 3β1,3 N-acetylglucosaminyltransferase (C3Gnt) are highly susceptible to inflammatory insults including dextran sodium sulphate (DSS) challenge[77]. Transgenic animals with alterations or lack of glycosylation in these mucins show increased colonisation by enteric pathogens than in wildtype mice[78, 79].

Increased bacterial colonisation of the epithelial layer often results in many common disorders including colon cancer and coeliac disease[80-82]. In humans, missense mutations that occur in the MUC2 gene have also been reported to result in aberrant MUC2 oligomerisation and subsequently increased susceptibility

to spontaneous ulcerative colitis[79]. Under normal conditions, goblet cells constitutively secrete mucins, however, mucin production has been reported to be upregulated by Toll-like receptor signalling to replenish the mucus degraded by bacteria or removed by peristalsis[83].

1.2.3 The intestinal epithelium

The intestinal epithelium is formed by a single-cell layer that acts as a barrier against the external environment[84]. In addition to its role in absorption of nutrient, metabolite and water, the intestinal epithelium regulates interactions between the luminal contents, such as the bacteria breaching through the mucus layer and the underlying immune system[85-91]. In the small intestine, the epithelium forms crypts and villi whereas in the colon epithelium only contains crypts which are lined largely with goblet cells[92]. At the base of these crypts are stem cells, which proliferate, differentiate into enterocytes, migrate to the villus tip, and eventually slough into the lumen via anoikis[93-95]**(Figure 6)**. This entire process results in the renewal of the epithelium every 5 days. Thus, theremarkable proliferative and self-regenerating properties of the GI epithelium, one of the highest renewal rates in the body[96], acts as one form of intestinal defence against injury[84, 97]. The gut microbiota influences intestinal epithelial cell differentiation, proliferation and apoptosis[98-100]. The turnover time of epithelial cells in the duodenum and ileum of conventionally raised mice is lower in GF mice [101, 102]. It was recently showed that the median protein half-life of epithelial cells in the small intestine was shorter than those in the colon[103]. The small intestine relies on fast passage time and high concentrations of antimicrobial peptides originating largely from the Paneth cells for its protection[104]. The colon relies more on a thick mucus layer for its protection, which demands an increased mucus synthesis capacity in the presence of bacteria, which is reflected in the higher protein turnover observed in the colonocytes[103]. Furthermore, the goblet cell-to-enterocyte ratio changes along the GI tract, with an estimated percentage of goblet cells in the human intestinal epithelium of approximately 4% in the duodenum, 6% in the jejunum, 12% in the ileum and 16% in the distal colon[105]. This gradual variation can be explained by the fact that, along the GI tract, the proportion of goblet cells increases proportionally to the increase in the number of microorganisms.

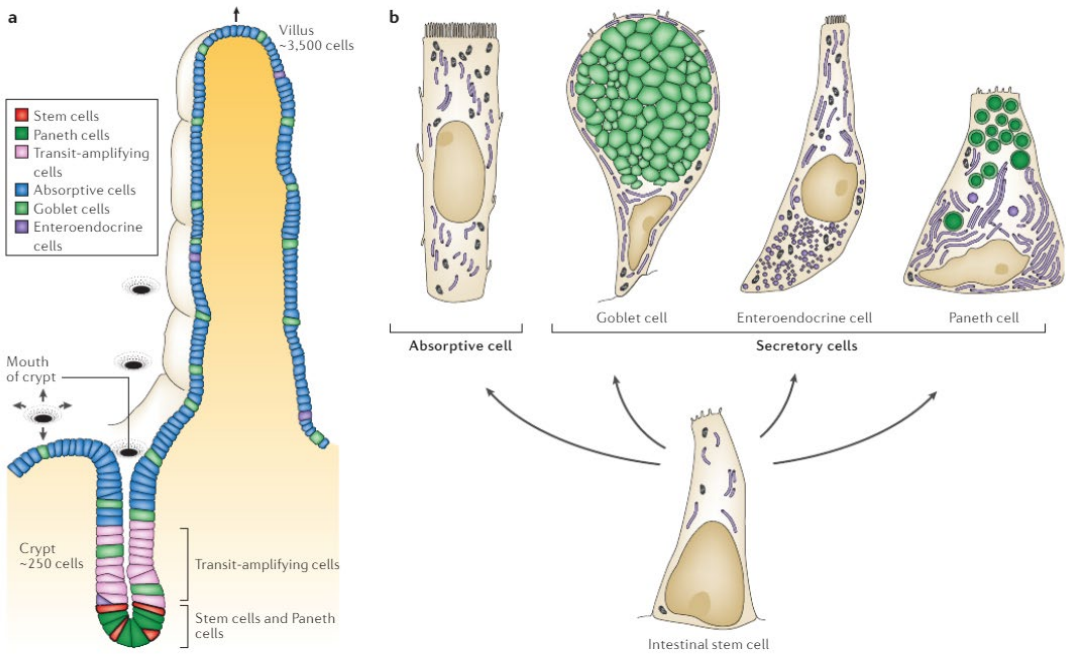

Figure 6: The distribution of epithelial cell types in mammalian small intestine.

(A) A villus with one of the crypts that contribute to renewal of its epithelium. In the colon there are no villi, but the organisation is otherwise similar. **(B)** There are four classes of terminally differentiated cells. Absorptive cells have a brush border on the apical surface. The other three classes are all secretory: goblet cells secrete mucus, enteroendocrine cells secrete various gut hormones and Paneth cells lie at the bottom of the crypts and secrete antibacterial proteins. Taken from Crosnier et. al., 2016.

Most cells found in the gut epithelium are absorptive enterocytes which are adapted for metabolic and digestive functions and secrete several hydrolytic enzymes[12, 106]. They are involved in the absorption of a range of dietary nutrients including amino acids, salts, sugars, lipids, and water[89]. Moreover, enterocytes can take up and process antigens by lysozyme degradation, before presenting them directly to T-cells to elicit an immune response[107]. The other specialised cells of the epithelial layer include enteroendocrine cells which are responsible for the release of hormones that control digestive function, as well as mucin secreting goblet cells and anti-microbial secreting Paneth cells which contribute to the physical and biochemical barrier to microbes[108,12]. The epithelium maintains its selective barrier function through the formation of complex protein-protein networks that mechanically link adjacent cells and seal the intercellular space[109].

1.3 Host secretory antimicrobials

1.3.1 Antimicrobial proteins

Most antimicrobial proteins are produced by Paneth cells and enterocytes in the small intestine[110-112] whereas in the large intestine the antimicrobial peptide gradient is reinforced by the mucus bi-layer[113]. Due to the absence of Paneth cells in the large intestine, antimicrobial peptides are not synthesised by cells that reside in colonic tissue[114, 115] but accumulate in association with colonic luminal contents[116]. This difference in antimicrobial production is believed to play a crucial role in shaping the distinct microbial profiles present in the small and large intestines.

Secreted IgA (sIgA), produced by plasma cells, migrate from lymphoid sites to the LP where they are translocated across the epithelium layer and deposited on the apical surface of the epithelial cells (**Figure 7**). Transcytosis of sIgA across intestinal epithelial cells (IECs) is mediated by the polymeric immunoglobulin receptor (pIgR)[117, 118]. Humans secrete approximately 66 $mg.kg^{-1}$ of sIgA into the intestinal lumen every day, reflecting the importance of sIgA in protecting the mucosal surface[119]. Binding of transcytosed sIgA to microbes on the luminal side of the epithelial barrier is believed to cause aggregation, which leads to slower diffusion and restricts microbe mobility towards the epithelial cell surface or induces phagocytosis of microbes that reach the epithelial cells[120]. Furthermore, sIgAs have been shown to bind intracellular pathogens in endosomes upon their transcytosis to the lumen[121] and to confer immune response capable of withstanding viral infections[122].

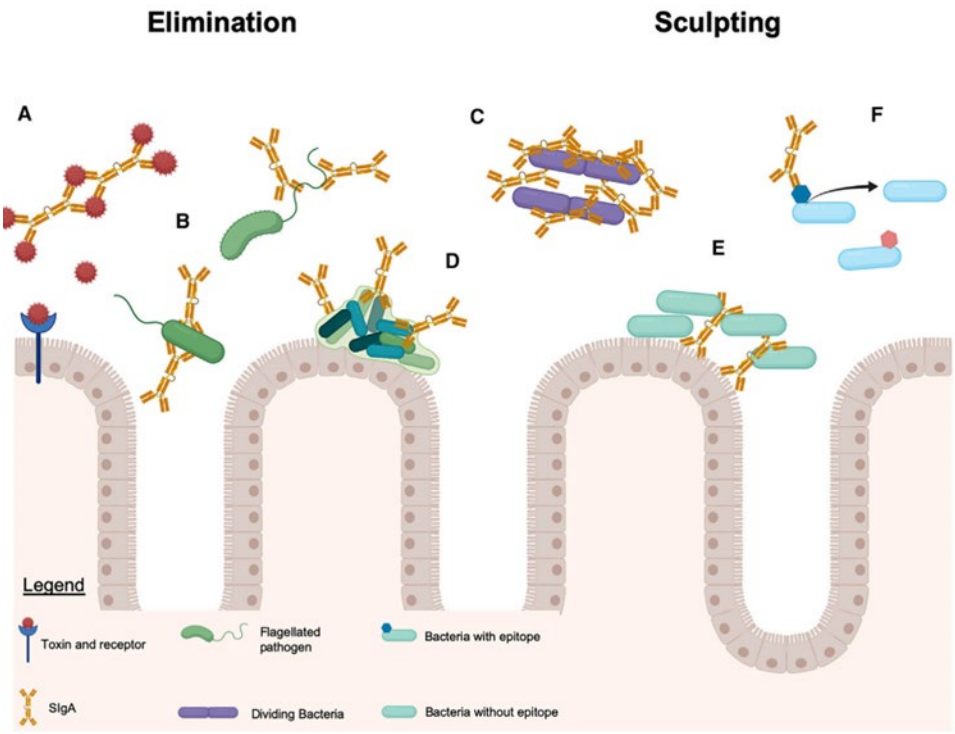

Figure 7: Proposed mechanisms by which SIgA influences gut microbes.

(A) SIgA eliminates toxins and/or neutralises microbial molecules by direct binding**. (B)** Directly binding to the pathogen and limiting motility and likely invasion. **(C)** Aggregation of rapidly dividing bacteria by enchained growth prevents over-population by proliferation while also limiting access to the epithelia. **(D)** Biofilms are often mechanisms by which microbes can adhere to surfaces and allow colonisation. SIgA can prevent biofilm formation, which would be predicted to prevent some organisms from colonising surfaces. **(E)** SIgA can preferentially bind to surface microbial molecules that anchor the microbe to the epithelial surface. **(F)** Microbes are known for the ability to sense environmental cues and respond by changing their gene expression patterns. SIgA binding of specific molecules on microbes might be sensed by the microbe such that binding results in down-regulation of that surface molecule. In this way, SIgA could fine-tune microbial gene expression to prevent the production of proteins that could be harmful to the host. Taken from Weis and Round., 2021.

The important role of sIgA in barrier function has been shown using B cell-deficient mice and mice lacking the immunoglobulin receptor involved in the transportation of sIgA to the lumen[123]. These knockout mice are prone to over stimulate innate responses after bacterial challenge. SIgAs have also been implicated in facilitating the uptake of microbes into IgA-inducing Peyer's patches, and inhibit interleukin (IL)-12 and induce IL-10 cytokine secretion *in vitro*, which results in the induction of T helper cell 2 (Th2) or regulatory T cell (Treg) responses[124]. These multiple functions of sIgA collectively reinforce the integrity of the intestinal barrier, lessen unnecessary proinflammatory responses, and thereby contribute to intestinal homeostasis.

Another key group of antimicrobial proteins encompasses enzymes such as lysozyme, secretory phospholipase A2 (sPLA2) and DMBT1 (deleted malignant brain tumours 1) that kill bacteria through an enzymatic degradation of essential cell membrane or cell wall components of microbes[125, 126]. Lysozyme, a 15 kDa single chain protein, is a glycosidase that functions by hydrolysing the β-1,4-glycosidic bonds between the *N*-acetylmuramic acid and *N*-acetylglucosamine (GlcNAc) moieties of peptidoglycan[127]. Lysozyme is, therefore, more effective against Gram-positive bacteria where the peptidoglycan is more accessible than against Gram-negative bacteria, where the peptidoglycan is covered by the outer membrane[128]. sPLA2 is another example of antimicrobial proteins that kill bacteria through an enzymatic activity. LsPLA2 is produced abundantly by Paneth cells, as for lysosyme, and catalyses the release of arachidonic acid from membrane phospholipids found on bacteria, thus compromising bacterial membrane integrity[129, 130].

Regenerating islet-derived 3 (REG3) proteins are key mammalian antimicrobial proteins that are expressed predominantly in the colon[131, 132]. Human REG3 proteins comprise a carbohydrate recognition domain (CRD) and a *N*-terminal secretion signal[133]. Three distinct classes of REG3, Reg3-α, -β, and -γ have been identified in mice[134] whereas only REG3-α and -γ have been identified in humans[134]. Human REG3-α, also known as hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP), has a murine homolog designated REG3β[132]. The antimicrobial proteins REG3β and REG3γ are secreted C-type lectins (**discussed in section 1.4.2**)[135] that are predominantly expressed in the GI tract, but not in the liver, under homeostatic conditions[136]. Importantly, REG3 recognition of peptidoglycan involves a unique mechanism that allows high-affinity binding to the extended carbohydrate chains of surface peptidoglycan but not to shorter, soluble peptidoglycan chains[136]. This allows selective binding to the bacterial surface while avoiding competitive inhibition by shorter peptidoglycan chains that are shed by bacteria and are thus abundant in the intestinal environment[133, 137]. REG3γ has bactericidal activity against Gram-positive

bacteria and helps maintain the spatial segregation of luminal bacteria and the intestinal epithelial surface[134, 135]. REG3β has bactericidal activity against Gram-negative bacteria and has been shown to protect mice against intestinal infection and dissemination of *Salmonella enteritidis*[138, 139].

Angiogenins are monomeric proteins that belong to the pancreatic ribonuclease superfamily[140, 141]. Although angiogenins were originally implicated in the growth of tumours, some members of the family such as Ang4 have been identified as Paneth-cell derived anti-microbial peptides important in epithelial host defense against gut-dwelling bacteria in the small intestine[142]. Importantly, the induction of Ang4 by commensal bacteria distinguishes it from other microbicidal proteins such as defensins which do not appear to be regulated by bacteria[142, 143].

1.3.2 Lectins

Lectins are a group of proteins of non-immune origin that can either be free or linked to cell surfaces[144]. Lectins have been shown to be involved in numerous biological processes including cell-cell interactions, signalling pathways, cell development, and immune responses[144]. Lectins, via their carbohydrate recognition domain (CRD), selectively bind distinct carbohydrate features of select populations of microbes[145]. Several CRDs comprise protein-bound cations, especially calcium ions, that plat a crucial role in the stabilisation of the CRD structure[146]. Furthermore, calcium ions present in the binding sites of C-type lectins, pentraxins, and intelectins facilitate carbohydrate recognition[146, 147]. Many lectins bind to their carbohydrate ligands through a "lock-and-key" binding mode, with little change in lectin conformation upon carbohydrate binding. Divalent calcium ions or other cations may therefore contribute to organise and rigidify the CRD and its binding site. This pre-organisation is thought to reduce the entropic penalty paid upon carbohydrate complexation.

1.3.2.1 Galectin-3

Galectins constitute a phylogenetically conserved family of lectins, ubiquitous in eukaryotes, including mammals, parazoa (sponges) and both protostome and deuterostome lineages of metazoans, and fungi [148-151]. Galectins are defined by the presence of a CRD composed by a shared consensus of approximately 130 amino acid sequence and by their high affinity for β-galactoside rich glycoconjugates. In mammals, 15 members of the galectin family have been identified to date. Based on their structural properties, galectins have been classified into 'prototype' (Gal-1, −2, −5, −7, −10, −11, −13, −14, and −15), 'chimaera' type (Gal-3), and 'tandem-repeat' type (Gal-4, −6, −8, −9 and −12) [152] (**Figure 8**). Prototype galectins only consist of one CRD per protein and are non-covalently linked homodimers. The chimaera galectins have a C-terminal CRD and an N-terminal domain rich in proline and glycine [153]. In

tandem repeat galectins, two CRDs are joined by a functional linker peptide. Galectins have been implicated in many pivotal roles including signalling, development, differentiation, cell-cell adhesion, cell-matrix interaction, growth regulation, apoptosis, RNA splicing, and cancer metastasis [154-156]. Several cell types, including epithelial cells, endothelial cells, and mucosal-associated immune cells are major sources of galectins in the intestine [157]. Galectins represent a branch of the lectin family whose members have antibacterial functions [158]. Gal-3 is a unique ~30 kDa chimeric type protein produced by various cells and particularly by epithelial and immune cells **(Figure 8b).** Gal-3 is detected in the cytoplasm and nucleus of the cells and is implicated in various cellular processes such as anti-apoptosis, cell proliferation, and pre-mRNA splicing [159]. Despite lacking a signal peptide, Gal-3 can be secreted via a nonclassical pathway and thus is found on the cell surface and/or in the extracellular space[160].

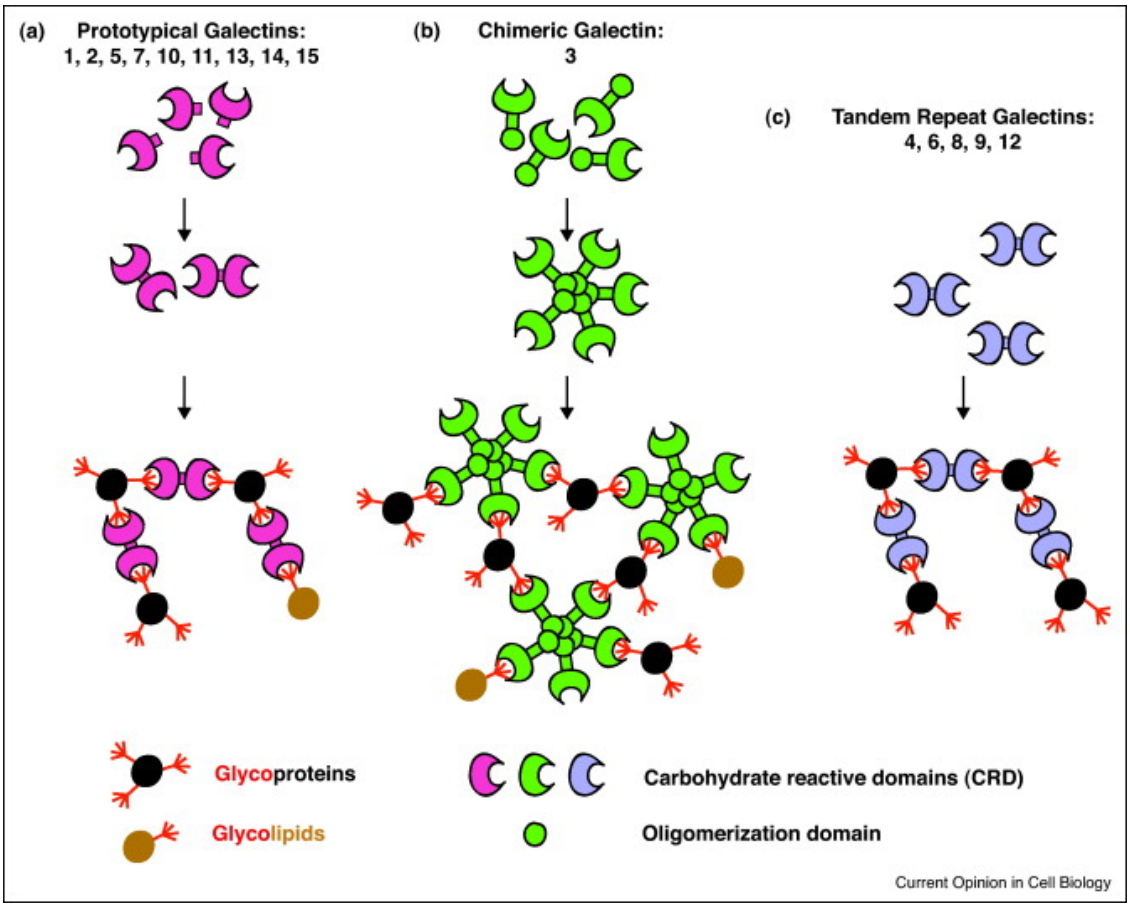

Figure 8: The galectin lattice.

Galectins are represented with their carbohydrate reactive domains (CRD) whose interactions with glycoproteins and glycolipids form a complex lattice at the cell surface. **(A)** Prototypical galectins 1, 2, 5, 7, 10, 11, 13, 14 and 15 have one CRD and can dimerize to form a cell surface lattice. **(B)** The chimeric Gal3 contains a CRD domain and non-lectin N-terminal domain responsible for its oligomerization (up to pentamers) forming a complex lattice with a different geometry than the one formed by prototypical galectins. **(C)** Similarly, to the prototypical galectins, the tandem repeat galectins 4, 6, 8, 9 and 12 have two CRDs and form a complex lattice. Taken from Boscheret.al., 2011

Secreted Gal-3 binds to a wide array of glycoproteins and glycolipids found on cell surfaces or extracellular matrix and plays biological roles such as in angiogenesis and cell adhesion [161, 162]. Moreover, due to their ability to form a pentamer, Gal-3 lectins can act as a bivalent or multivalent receptor, and cross-link cell surface glycoconjugates, which, like many other receptor-ligand systems, can trigger a cascade of transmembrane signalling events [163, 164]. Upregulation of Gal-3 in transformed and metastatic cell lines have been reported in various studies[166]. In the GI tract, Gal-3 is predominately found in the villus, and has been recently shown to differently recognise intestinal mucins (such as MUC2) with different *O*-glycosylation profiles[165]. Glycans associated with MUC2 imprinted DCs with anti-inflammatory properties by assembling a Gal-3-Dectin-1-FcγRIIB receptor complex that activated β-catenin. This transcription factor interfered with DC expression of inflammatory but not tolerogenic cytokines by inhibiting gene transcription through nuclear factor κB. MUC2 induced additional conditioning signals in intestinal epithelial cells[166]. Gal-3 has also been shown to interact with lipopolysaccharide (LPS) of pathogenic bacteria such as *Klebsiella pneumoniae, Pseudomonas aeruginosa, Neisseria gonorrhoeae* and *Helicobacter pylori* via the binding to the β-galactoside glycans of the outer core [167]. However, Gal-3 can also bind other structures as demonstrated by its interaction with *Salmonella minnesota* LPS which is devoid of β-galactosides[168]. Gal-3 interaction with pathogens often results in reduced or exaggerated states of endotoxic shock, or increased adhesion to host tissues[169, 170]. Recently, screening of commensal bacteria for their ability to bind with Gal-3 showed a higher affinity of Gal-3 for two specific strains of *Bifidobacterium longum*, requiring the full-length Gal-3 for enhanced activity[171]. These studies highlight the ability of pathogenic and commensal bacteria to capitalise on the presence of Gal-3 to augment their ability to inhabit the intestinal environment.

1.3.2.2. Intelectins

Intelectins (IntLs) are calcium-dependent galactose binding lectins which exist as homo-oligomers of 35-kDa monomers[172]. Despite lacking a calcium-dependent C-type lectin sequence motif, IntLs are reported to function as calcium ion-dependent lectins [172] **(Figure 9)**. IntLs contain a fibrinogen-like domain and have been proposed to function like ficolins, a family of innate immune lectins[173-175]. First identified in African clawed frogs, IntL homologs have since been reported in many other amphibians, fishes and mammals[176]. There are two types of IntLs in humans, human IntL-1 (hIntL-1) and human IntL-2 (hIntL-2), which differ based on their sugar recognition properties[145].

HIntL-1, also known as omentin or intestinal lactoferrin receptor, is expressed by various tissues including in the intestine by Paneth cells, enterocytes and goblet cells [177]. The exact role of this protein is

not clear, but hIntL-1 has been implicated in metabolic human disorders such as diabetes and cardiac hypertrophy[178]. HIntL-1 has been reported to bind D-galactofuranose moieties including ribofuranose and a β-Galf–containing disaccharide[177]. The monosaccharide Galf is present in cell-surface glycans produced by many microorganisms, but the biosynthetic enzymes that mediate Galf incorporation are absent in humans, which synthesise only the six-membered ring form, galactopyranose[178]. The presence of Galf in microbial but not human glycans is an example of phylogenetic glycan differences. In principle, recognition of monosaccharide residues found exclusively on microbes could be used by the innate immune system to differentiate human glycan epitopes from microbes. For example, hIntL-1 has been shown to recognise *Mycobacterium bovis* bacillus Calmette-Guérin[179], *Vibrio cholerae* and other enteric bacterial pathogen[180]. HIntL-1 can also utilise a bound Ca^{2+} to coordinate terminal exocyclic 1,2-diols. N-acetylneuraminic acid (Neu5Ac), a sialic acid widespread in human glycans, has an exocyclic 1,2-diol but does not bind hIntL-1, probably owing to unfavourable steric and electronic effects[178]. HIntL-1 recognised only *Streptococcus pneumoniae* serotypes that display surface glycans with terminal 1,2-diol groups. HIntL-1 expression is also increased rapidly after gastrointestinal nematode parasite infection in mice and sheep[181-183]. Their specificity suggests a role for the intelectins as host defence molecules in the small intestine.

HIntL-2 is likely a Ca^{2+} dependent carbohydrate-binding lectin whose expression, unlike hIntL-1, is restricted to intestinal goblet and Paneth cells[184]. Not much is known about hIntL-2, but it appears to contribute to the innate immune response against fungal/parasitic infection[178]. Mouse IntL-2 (mIntL-2) was found to be highly upregulated in intestinal epithelium following infection of mice with *Trichinella spiralis*[184].

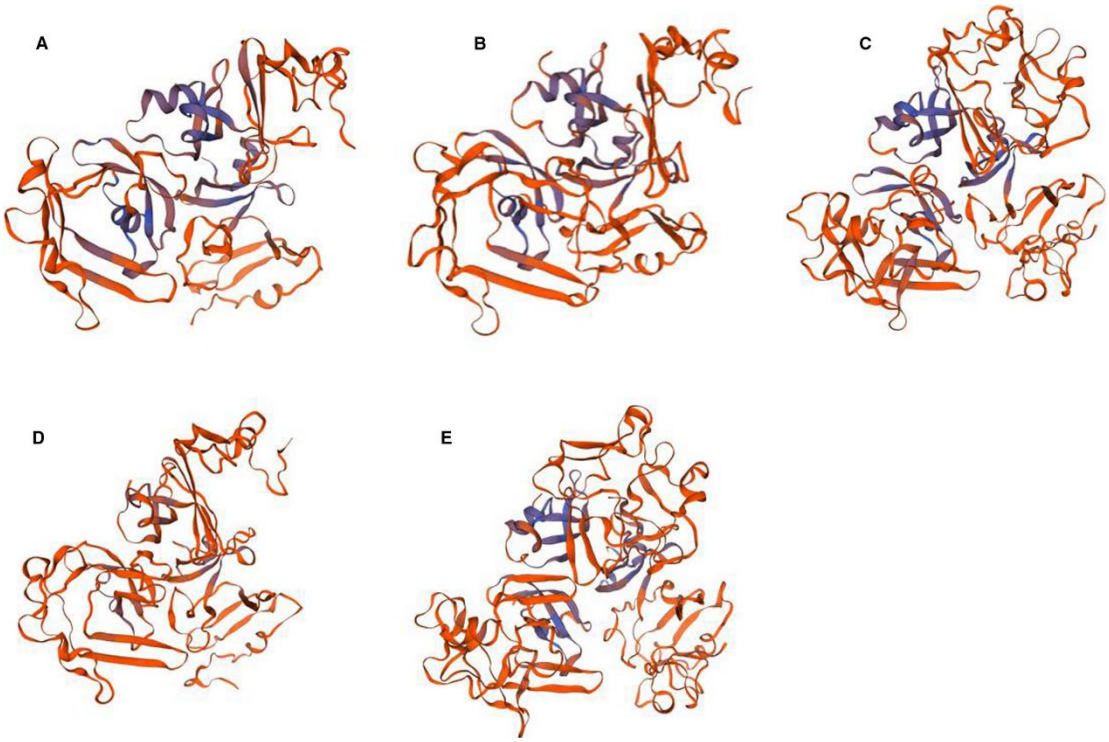

Figure 9: Predicted 3D structures of intelectins by homologous modelling.

(A) Homo sapiens hILTN1, **(B)** Danio rerio zITLN1, **(C)** Xenopus embryonic epidermal lectin, **(D)** Ciona intestinalis ENSCING00000009653 and **(E)** Ictalurus punctatus ITLN. Taken from Chen et. al., 2020

1.4 Lamina propria DCs

Although bacteria are largely confined to the luminal side of the epithelial barrier, the sheer number of intestinal bacteria makes occasional breach inevitable. Typically, commensal microorganisms that penetrate the intestinal epithelial cell barrier are phagocytosed and eliminated by LP resident macrophages or dendritic cells (DCs)[185]. Epithelium-associated (EA)-DCs can also actively participate in antigen capture across the intestinal epithelium as they can extend protrusions directly into the lumen for bacterial sampling[186]. The extension of trans-epithelial dendrites (TEDs) varies depending on the segment of the intestine that is analysed[187]. The proximal intestine (jejunum, duodenum, and proximal) displays a higher number of TEDs than the terminal ileum under steady-state conditions. TEDs are dependent on the gut microbiota as antibiotic treatment drastically reduces their number[187].

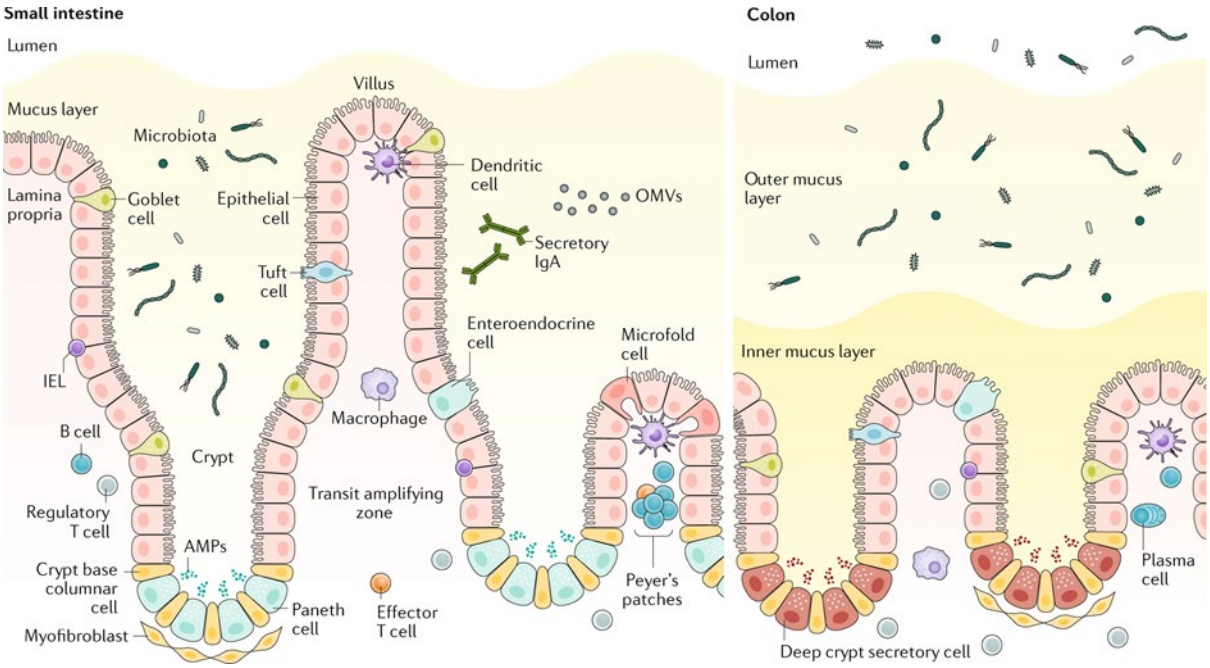

Figure 10: General overview of mucosal immunity to intestinal pathogens and commensal microorganisms.

The GI mucosa is separated from the environment by a single layer of IECs that provides a physical and functional barrier. Beneath the IECs, stromal cells (myofibroblasts), B cells and IgA-producing plasma cells, macrophages, DCs and T cells dwell in the LP, reinforcing the epithelial barrier by sampling luminal contents and maintaining a hyporesponsive state. Regional lymphoid structures, such as Peyer's (small intestine), caecum and colon patches, and the solitary isolated lymphoid tissues are overlaid by a specialised epithelium, known as follicle-associated epithelium, where microfold cells capture antigens and release them into the subepithelial dome. In the colon, the presence of a firm inner mucus layer reduces exposure to microorganisms. However, the microorganism-associated molecular patterns embedded in outer membrane vesicles can eventually reach the IECs. Taken from Perez-Lopez et. al., 2016.

DCs are located throughout the body to capture and internalise foreign antigens, and subsequently, process and present peptides in associating with major histocompatibility complex (MHC) class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively[188]. However, antigen presentation is not sufficient to trigger potent T cell response against invading microbes. For an effective adaptive immune response, $CD4^+$ T cells must differentiate into distinct T helper cell subsets depending on the source of the antigen; Th1 cells produce proinflammatory cytokines such as interferon-γ (IFNγ), which triggers macrophage activation to clear intracellular pathogens, TH2 cells produce IL-4, IL-6 and IL-10 to trigger humoral immune responses against helminths, and IL-17-secreting Th17 cells which are involved in the mobilisation of macrophages and DCs to fight extracellular parasites and bacteria[189]. Moreover, regulatory T cells are required to control the activity of effector TH cells to avoid aberrant immune activation[190-192]. Thus, DCs are involved in the translation of information about the invading bacteria into a cytokine gene expression profile that directs the correct TH cell differentiation pathway[193]. Pathogen recognition is essential to the induction of a systemic immune response. Although the range of pathogens is immense, many microbes share similar properties and structures known as pathogen-associated molecular patterns (PAMPs), which mediate their recognition by the host[194, 195]. Like many antigen presenting cells (APCs), DCs express various pattern recognition receptors (PRRs) that recognise PAMPs to induce an immune response[196]. These PRRs include, but are not limited to, the archetypical Toll-like receptors (TLRs), as well as non-TLRs such as intracellular nucleotide-binding domain and leucine-rich-repeat-containing family (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors and C-type lectin receptors (CTLs)[197].

1.4.1. TLR

In humans and mice, 10 and 13 TLRs have been identified, respectively, with TLR1–TLR9 being conserved in both species (**Figure 11**)[194, 198]. All TLRs share a similar domain organisation, as each TLRs is a type I transmembrane proteins with N-terminal ectodomains comprising leucine-rich repeats, a transmembrane domain, and a cytosolic TIR domain required for downstream signal transduction[199]. Mammalian TLRs that are found in the plasma membrane include those that detect microbial cell surface components, such as TLR4, which detects LPS[200], TLR5 (flagellin)[201, 202], and TLRs 1, 2, and 6 (bacterial lipoproteins)[203-205]. TLRs found in endosomes detect nucleic acids such as TLR3 (double-stranded RNA)[206], TLR7 and 8 (single stranded RNA)[207-209], TLR9 (unmethylated CpG containing single stranded DNA)[210], and TLR13 (bacterial ribosomal RNA)[211]. This complex collection of PAMP-PRR interactions allows multiple members of the TLR family to detect individual microbial cells. Disruption of

TLR signalling has been shown to contribute to a myriad of chronic inflammatory diseases including sepsis, asthma, atherosclerosis, rheumatoid arthritis and systemic lupus erythematosus[212].

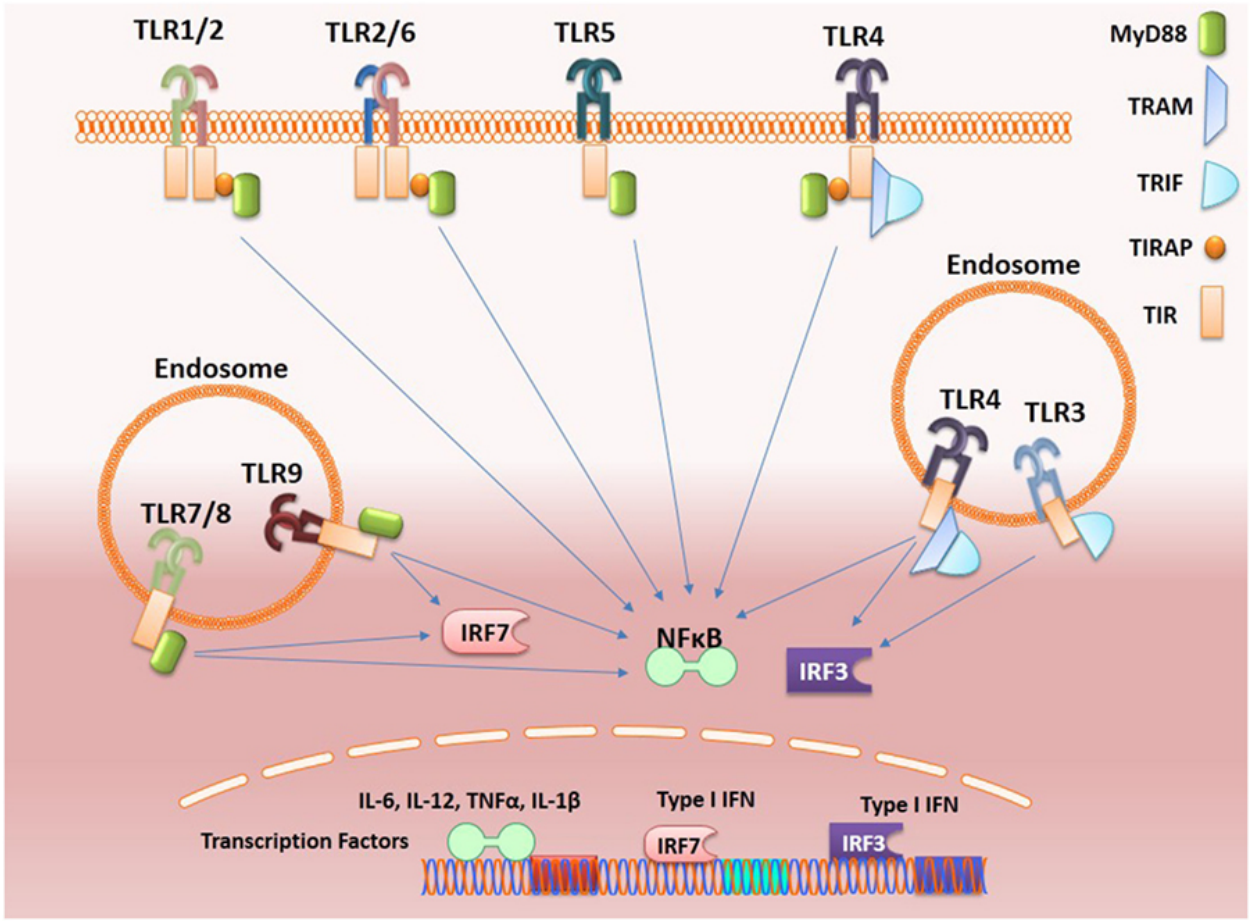

Figure 11: Toll-like receptors signalling.

Activation of TLRs can proceed through MyD88-dependent or TRIF-dependent pathways. Most of the TLRs form homodimers upon activation while TLR2 can also form heterodimers with either TLR6 or TLR1. These signals culminating in the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon-regulatory factors (IRFs), which induce, respectively, the production of inflammatory cytokines and type 1 interferon (IFNs). Activation of endosomal TLRs (TLR7 and TLR9) via MyD88 activates NF-κB and also IRF7 leading, respectively, to the production of inflammatory cytokines and type-1 IFNs, while the adaptor protein TRIF is recruited by the endosome-localized receptors TLR3 and toll-like receptor-4 (TLR4). TLR3 can interact directly with TRIF, while the TLR4–TRIF interaction requires the bridging to adaptor molecule TRAM, and both activate IRF3 that induce the production of type I IFNs. Taken from Zakeri and Russo, 2018.

1.4.2 C-type lectins

C-type lectins (CTLs) are an important class of PRRs that are involved in the induction of pathogen-specific gene expression profiles, either by modulating TLR signalling or by directly inducing gene expression[213]. Upon injury or damage of the epithelium, CTLs also play a crucial role in the host-microbiota interaction in the gut[214]. CTLs are a large family of proteins originally named for their ability to bind sugars in a calcium ion-dependent manner[215, 216]. CTLs consist of a distinct CRD protein fold which is maintained through disulphide bridges between conserved cysteine residues. This family has been divided into 17 groups based on the organisation of their CRDs and can be functionally defined as either classical or non-classical. Classical C-type lectins contain conserved residues in their CRDs which are responsible for forming Ca^{2+} binding sites and also generally contain conserved motifs which typically bind carbohydrate ligands, such as the EPN amino acid triplet which binds mannose-type carbohydrates or the QPD triplet which binds galactose-type carbohydrates. Non-classical C-type lectins or lectin-like receptors generally do not contain these residues and are more likely to, but do not necessarily, bind non-carbohydrate ligands, such as those encoded by the Natural killer-gene complex (NKC). Although they share structural homology, C-type lectins usually differ significantly in the types of glycans that they recognise with high affinity[146]. CTLs recognise an array of endogenous and exogenous glycosylated ligands found on fungi, bacteria, parasites, driving both innate and adaptive immunity[217, 218].

1.4.1.1 Dendritic cell-associated C-type lectin 1

Dendritic cell-associated C-type lectin 1 (Dectin-1) (also known as Clec7a) is a transmembrane protein expressed mainly on myeloid cells, including macrophages, and other mononuclear cells, as well as a subpopulation of T cells[219]. Dectin-1 is regarded as the major mammalian cell surface receptor for β-1,3 glucan and β-1,6 glucan, which are carbohydrates widely expressed on the cell wall of many fungal organisms such as *Pneumocystis carinii* or *Candida albicans or Aspergillus fumigatus*[220]. However, Dectin-1 can also recognise a number of mycobacterial species, even though β-glucans are absent from mycobacteria and the ligands mediating this recognition have so far remained elusive[221-224]. The interaction between Dectin-1 and *Salmonella typhimurium* induces peptide antigen presentation to T cells and subsequent immune responses[225]. Furthermore, Dectin-1 was found to be crucial in generating pro-inflammatory immune response against a non-typeable *Haemophilus influenza in vitro*[226]. Dectin-1 suppression has been shown to improved intestinal inflammation in mice[227]. This study revealed that abolishing signalling via Dectin-1 led to influence AMP production and facilitate the expansion of the gut commensal *Lactobacilli* that are protective during colitis[227].

Dectin-1 has a similar structure to the other members of the NK-cell-receptor-like C-type lectin family, with two notable exceptions (**Figure 12**). First, Dectin-1 lacks cysteine residues in its stalk region[228], in agreement with the lack of evidence for oligomerisation and the ability for the receptor monomer to be functional *in vitro*[229]. Second, Dectin-1 contains an immunoreceptor tyrosine-based activation motif (ITAM)-like motif in its cytoplasmic tail[228], which is involved in cellular activation[202, 230, 231].

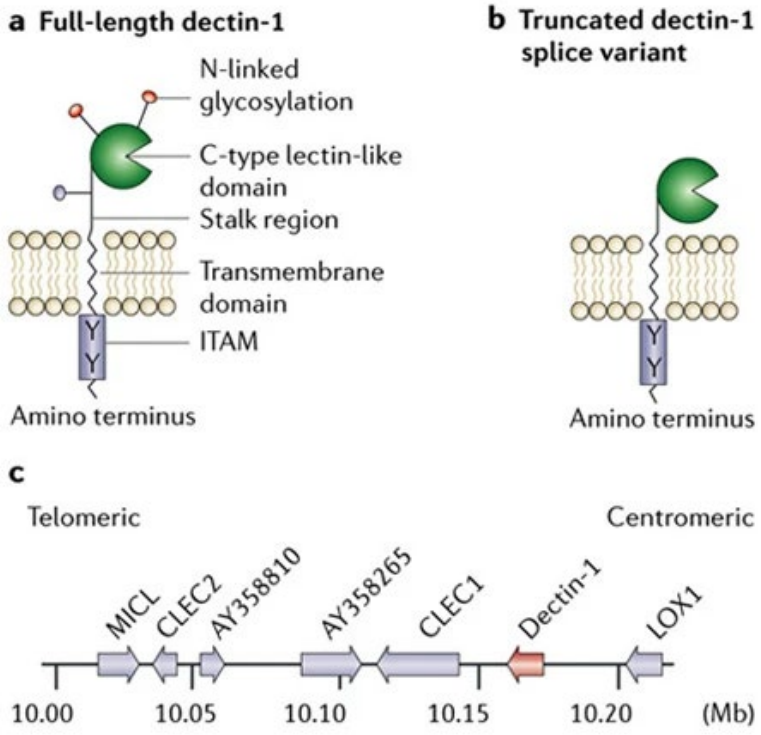

Figure 12: Dectin-1 structure and genomic localisation within the myeloid-cell-expressed natural killer (NK)-cell-receptor-like C-type lectin cluster.

(A) Dectin-1 is a type II transmembrane NK-cell-receptor-like C-type lectin with a single extracellular C-type lectin-like domain (carbohydrate-recognition domain, CRD), which recognizes β-glucans and an endogenous undefined ligand on T cells. (**B)** A splice variant of dectin-1 lacking the stalk region that arises due to alternative splicing. Although this has only been conclusively shown for the human receptor, it might also occur in mice. **(C)**| Dectin-1 is one member of a cluster of NK-cell-receptor-like C-type lectins found in the NK complex on chromosome 12 in humans (chromosome 6 in mice). Taken from Gordon et. al., 2006.

The activation of Dectin-1 by a ligand can lead to numerous downstream cellular responses, including the expression of cytokines such as TNF-α, IL-2, IL-10 and IL-12, and CXCL2[202, 230, 231]. Dectin-1-mediated activation can also induce phagocytosis[232] and respiratory burst[233]. Furthermore, Dectin-1 signalling has been shown to orchestrate adaptive immunity. DCs activation by Dectin-1 agonists are known to lead to the differentiating naïve $CD4^+$ T cells to a T helper- (Th-)1 or Th17 phenotype both *in vitro* and *in vivo*[234]. Dectin-1-activated DCs can also induce the maturation and proliferation of $CD8^+$ T cells *in vitro*[235]. In addition, the Dectin-1 agonist curdlan was found to act as an adjuvant for cytotoxic T lymphocyte cross-priming *in vivo*, which elicited potent responses capable of protecting mice from experimental tumour challenges[235]. Thus, Dectin-1 signalling serves as a receptor that generates appropriate adaptive responses following immune recognition[236].

Dectin-1 does not appear to initiate protective responses in isolation but acts synergistically with other receptors such as TLRs. For instance, Dectin-1 stimulation was found to augment TLR-2-mediated production of cytokines in murine macrophages and DCs[202, 230]. In addition, stimulation of human peripheral blood mononuclear cells (PBMCs) with a Dectin-1 ligand, as well as ligands for TLR-2 or -4, led to a synergistic increase in TNF-α production compared with Dectin-1 stimulation alone[237]. Shin et al. extended this investigation to mycobacteria by infecting murine macrophages with *Mycobacterium abscessus* (Mab), an environmental non-tuberculous Mycobacterium that can cause opportunistic infections in humans[238]. Mab stimulation of macrophages initiated a physical co-localisation between Dectin-1 and TLR-2 that was required for pro-inflammatory cytokine production[238] although the mechanisms underlying this apparent interaction remain to be elucidated[239, 240]. In contrast to these findings, a study by Rothfuchs et al. showed that Dectin-1 inhibition significantly reduced the production of IL-12p40 by DCs lacking TLR-2[222], suggesting that Dectin-1 signalling is not necessarily dependent on TLR-2. These seemingly conflicting results may be due to the different microorganisms and stimuli investigated on different cell types.

1.4.1.2 Dendritic cell-associated C-type lectin 2

Dendritic cell-associated C-type lectin 2 (Dectin-2) is the best characterised member of the C-type lectin receptor family. Dectin-2 is overexpressed in myeloid leukaemia mouse model and macrophages, neutrophils and pluripotent myeloid precursors[241]. Although originally proposed to be Langerhans cell specific, this receptor was shown to be expressed predominantly in tissue macrophages, DCs and at a low level on Langerhans cells (tissue-resident macrophages) and peripheral blood monocytes, where expression levels could be transiently increased upon induction of inflammation[242].

Dectin-2 is predicted to have mannose binding activity mediated by the presence of an EPN (Glu-Pro-Asn) tripeptide motif in its CRD. The CRD of Dectin-2 was shown to recognise zymosan and numerous pathogens including *Candida albicans*, *Saccharomyces cerevisiae*, *Mycobacterium tuberculosis*, *Microsporum audounii*, *Trichophyton rubrum Paracoccoides brasiliensis*, *Histoplasma capsulatum* and capsule-deficient *Cryptococcus neoformans*[243-245]. Although the level of binding to these pathogens differed greatly, binding was inhibited by chelation of Ca^{2+} or competition with mannose[243, 246]. Additionally, screening of a pathogen glycan microarray with Dectin-2 confirmed its specificity for high-mannose structures[243].

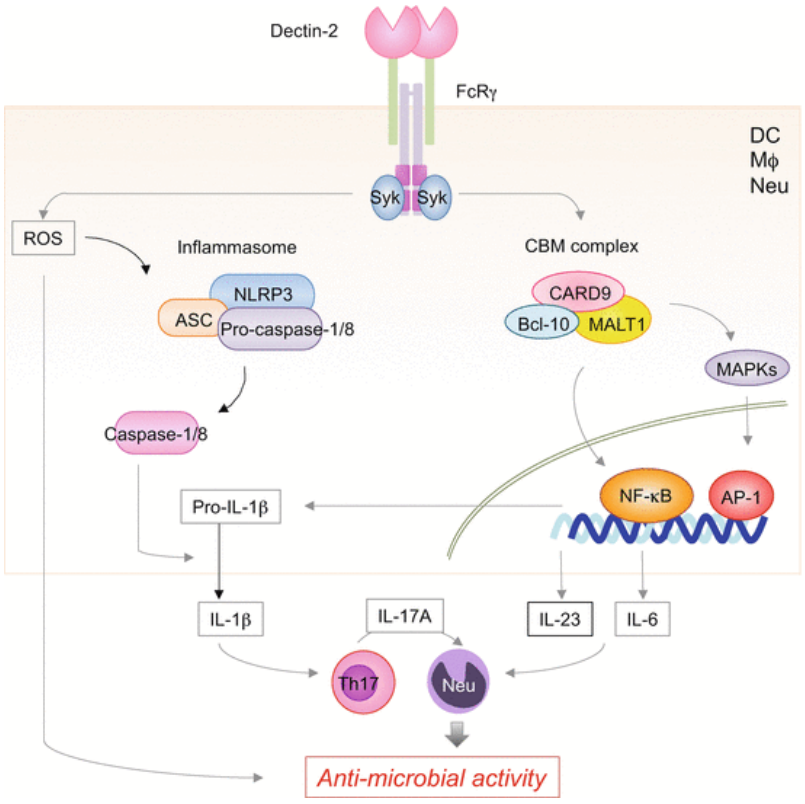

Figure 13: Cell signalling and immune response of Dectin-2.

Upon ligand binding, Dectin-2 recruits phosphorylated Syk to ITAM of the FcRγ, leading to activation of the CARD9–BCL10–MALT1 (CBM) complex. At the same time, reactive oxygen species (ROS) production is induced in a Syk-dependent manner, resulting in the direct killing of pathogens and activation of the NLRP3 inflammasome. The CBM complex activates NF-κB, which induces the production of cytokines such as pro-IL-1β, IL-6, and IL-23. In contrast, the NLRP3 inflammasome activates caspase 1 and/or caspase 8 to process pro-IL-1β into mature IL-1β. IL-1β, IL-6, and IL-23 preferentially induce the differentiation of Th17 cells, which play an important role in the host defence against microbes by recruiting neutrophils. Taken from Yabe and Saijo 2016.

The C-terminal portion of Dectin-2 encodes the extracellular region and the N-terminal portion encodes the cytoplasmic region of the receptor[247]. This protein is encoded by six exons and has a single CRD in the extracellular region, a stalk region, a transmembrane region, and a short cytoplasmic domain with no known signalling motif[245].

Dectin-2 associates with an adapter molecule, Fc receptor γ chain to transduce its signalling[246]. Although Dectin-2 contains an arginine residue, which often mediates associations with immunoreceptor tyrosine-based activation motif containing adaptor molecules in the transmembrane region, the interaction between these two molecules is not dependent on the arginine residue unlike other FcRγ-coupled receptors, but instead requires the cytoplasmic tail of Dectin-2[246]. As Dectin-2 has a conserved cysteine residue in its stalk region, which can form disulphide-linked homodimers, this receptor is expected to form homodimers upon ligand recognition[248] **(Figure 14).** It is possible that Dectin-2 recognises an endogenous ligand that is not a carbohydrate, perhaps via an alternative binding site to that which recognises fungi, as has been reported for other C-type lectins, such as Dectin-1[229]. Dectin-2 has also been shown to play a role in response to allergens[249]. Dectin-2 on bone-marrow-derived DCs (BMDCs) was able to bind to extracts from house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) and *Aspergillus fumigatus* in a mannose-dependent manner[249]. Stimulation of mast cells co-expressing Dectin-2 and FcRγ chain with these extracts resulted in the production of cysteinyl leukotrienes, proinflammatory lipid mediators which are not produced by untransfected cells[249]. Additionally, in primary BMDCs, signalling by Dectin-2 to produce cysteinyl leukotriene in response to the extracts was dependent on Syk kinase and FcRγ chain, and lentiviral knockdown of the receptor significantly reduced this activity[249].

Interestingly, some other C-type lectins have redundant biological functions to Dectin-2. For example, the macrophage C-type lectin (MCL, Dectin-3) senses the same ligand *C. albicans* α-mannan, as Dectin-2, and, more importantly, Dectin-3 and Dectin-2 form heterodimers as well as homodimers[250]. On the other hand, MCL also stabilises Mincle (an innate immune receptor) expression, and both receptors sense mycobacterial TDM[251, 252]. Furthermore, Dectin-3 has also been shown to recognise α-mannans on the surfaces of *C. albicans* hyphae and induced NF-kB activation. Compared to their respective homodimers, Dectin-3 and Dectin-2 heterodimers bound α-mannans more effectively, leading to potent inflammatory responses against fungal infections.

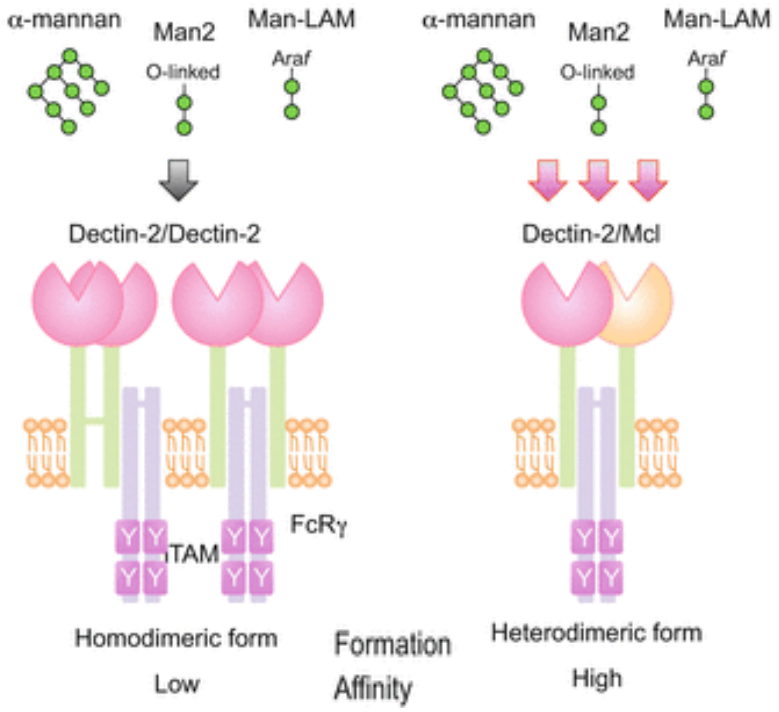

Figure 14: C-Type lectin receptors Dectin-2 and Mincle form a heterodimeric pattern-recognition receptor for host defence against fungal infection.

Dectin-2 senses α-mannosylated chains and initiates cellular responses through association with the Fc receptor γ chain (FcRγ), which contains immunoreceptor tyrosine-based activation motif (ITAMs). Dectin-2 is presented as a homodimer through a disulfide bond or in association with FcRγ, whereas it forms a heterodimer with MCL linked by FcRγ. The heterodimeric complex has relatively strong affinity to pathogen-associated molecular patterns (PAMPs) in comparison with the homodimeric Complex. Taken from Yabe and Saijo, 2016.

Dectin-2 has been shown to be an important receptor for the generation of the Th7-like adaptive immune response, coordinating the Th1-like responses together with Dectin-1[244]. Dectin-2 KO mice are more susceptible to *Candida glabrata* infections, showing a defective fungal clearance[244]. The increased susceptibility to infection was accompanied by lower production of Th1 and Th17-derived cytokines by splenocytes of Dectin-2 KO mice[244]. It is worth noting that Dectin-2 has also been implicated in protecting the host by dampening the excessive inflammatory responses[253]. It was reported that putative Dectin-2 ligand was expressed on regulatory T cells and blockade of Dectin-2-mediated signalling reduced immune tolerance[253,254].

1.4.1.3 DC-specific ICAM-3–grabbing non-integrin

DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN), another member of the family of Ca^{2+} dependent CTLs, recognises high mannose- and fucose-substituted glycans[255]. In humans, DC-SIGN can be found on intestinal DCs and macrophages in peripheral tissues like the placenta and lung, as well as mature DCs in lymphoid tissues[256-258], but not on other APC subsets, including plasmacytoid DCs or Langerhans cells[259-261]. In addition to pathogen recognition, DC-SIGN mediates antigen internalisation, processing, and presentation of antigens to T cells, which are functional hallmarks of APCs[262]. DC-SIGN is present on the cell surface as a tetramer, and therefore multivalent presentation of its carbohydrate ligand is favoured for high affinity binding[263] (**Figure 15**). Of the eight identified mouse homologs, CD209b (or SIGNR1) has been most widely investigated and shares glycan-binding specificity with DC-SIGN, including Lewis antigens[262]. DC-SIGN-mediated interaction between *Lactiplantibacillus plantarum* and human DCs has been shown to improve DC maturation, regulate the secretion of anti-inflammatory and pro-inflammatory cytokines, and induce the polarisation of interlectin-4-producing T cells[264]. Furthermore, *Lactobacillus rhamnosus* (JB-1) interaction with DC-SIGN was found to be crucial preventing excessive inflammatory responses against this non-pathogenic microbe is to suppress human DC activation *in vitro*[265]. In addition, DC-SIGN interaction with the major S layer protein, SlpA of *L. acidophilus* NCFM was shown to be involved in the modulation of human derived DCs and T cells functions[266]. In mice, SIGNR1 depletion have been shown to lead to susceptibility to infection by uropathogenic *E. coli*, resulting in prolonged bacterial persistence[267]. SIGNR-I has also been shown to interaction with gut commensal bacteria such as *L. reuteri* [268].

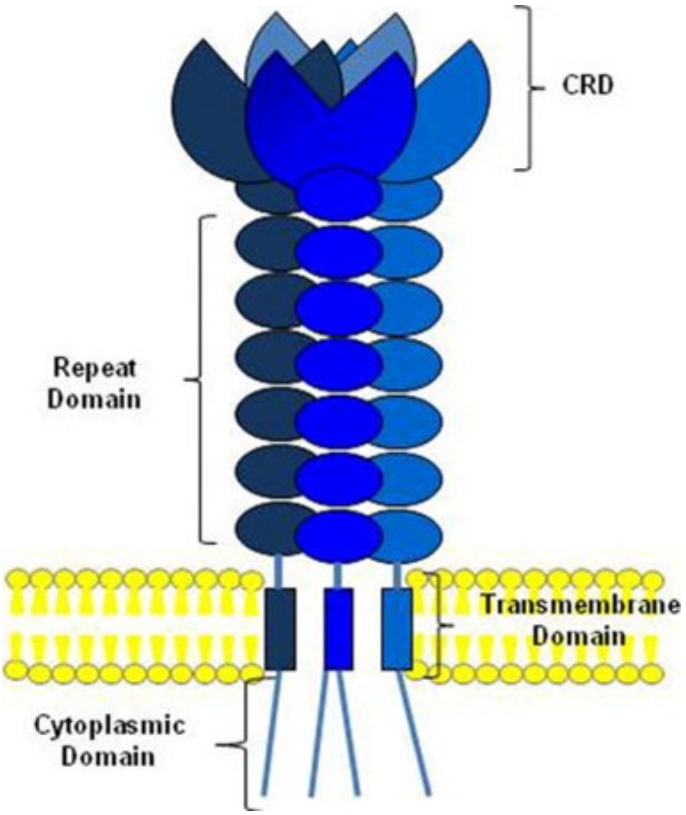

Figure 15: Schematic representation of tetrameric form of DC-SIGN.

DC-SIGN receptors are organised into three structurally distinct regions: an intracytoplasmatic tail domain responsible for internalisation and signal transduction, a transmembrane domain and finally an extracellular domain, which is further divided into two structures, the neck repeat region and the CRD domain**.** Taken from Da Silva et. al., 2018.

2. THE INTESTINAL MICROBIOTA

2.1 Overview

The human body is colonised by a multitude of microbes that have co-evolved with their hosts to form a symbiotic relationship. The mammalian intestinal microbiota or gut microbiota, which comprises bacteria, archaea, eukaryotes and viruses, is the most densely populated microbial communities in the body. The GI tract comprises several microbial niches which are dependent of many environmental, physiochemical and genetic factors, and thus differ greatly between individuals, as well as within a single host[2]. Molecular techniques reveal approximately 500–1000 species within several major phyla[269,270]. The concentration of bacteria in the human GI tract has been estimated to reach 10^2–10^4 bacterial cells mL^{-1} in the stomach and upper two-thirds of the small intestine[271], 10^7–10^8 bacteria mL^{-1} in the ileum and 10^{10}–10^{11} bacteria mL^{-1} in the colon [271].

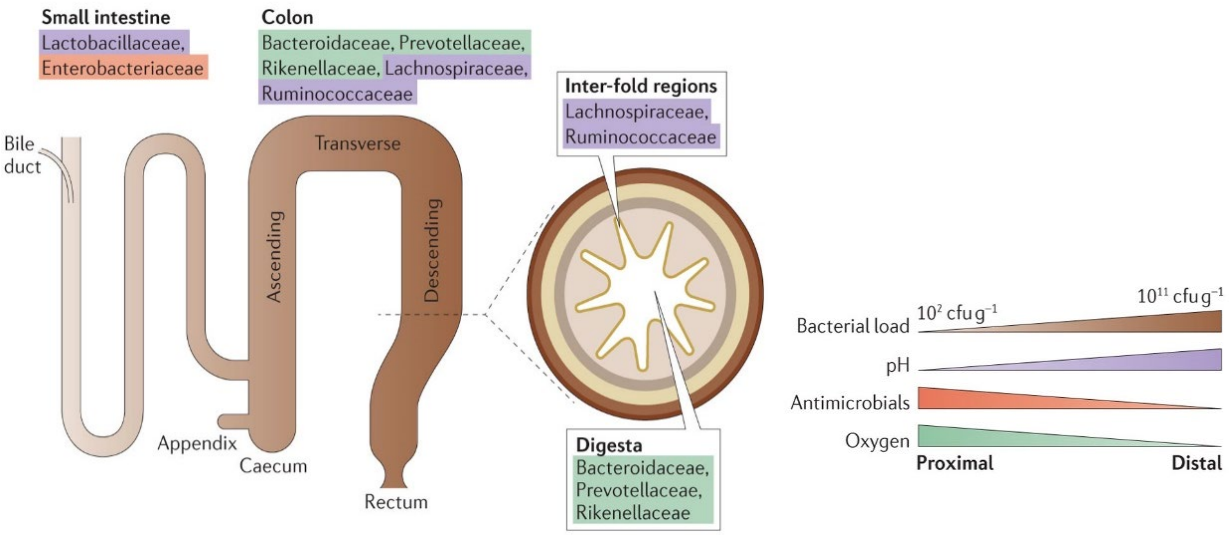

Figure 16: Bacteria distribution along and across the lower GI tract.

Changes in physiochemical conditions along the gut (pH, antimicrobial peptides and oxygen lead to a regional specificity in bacterial family dominance, with colon carrying the higher bacterial load when compared to the small intestine. The cross-section shows the bacterial family dominance in the digesta and the inter-fold regions of the lumen. Cfu = colony-forming units. Taken from Donaldson et. al., 2015.

The human gut microbiota is dominated by members of only four divisions, Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes[272, 273]. While related individuals share some similarity in terms of microbiota composition, a core microbiota, i.e., a group of microbial species that are common across the population, is considered unlikely to exist. In addition, microbial communities that differ in terms of composition may share some degree of functional redundancy, and this has led to the hypothesis of a core microbiome, which includes genes that perform conserved functions across all microbiota[274]. In addition to inter-individual variations, the gut microbiota varies along the length of the GI tract but also cross-sectionally of the mucosal surface[104]. Bacteria colonise the lumen, the outer mucus layer, and some adhere to the mucosal surface or can be found in the intestinal crypts[275-277] **(Figure 16).**

2.2 Factors affecting the microbiota composition

Colonisation of the infant's GI tract begins at birth[278], although some studies suggested that human intestinal microbiota could be seeded before birth[279], which remains a matter of debate[280]. Microbe colonisation sets the stage for the lifelong, relatively stable microbiome, which shapes the development of the GI tract and intestinal immune system, as well as future composition of the adult microbiome[278]. Although the adult microbiota is considered relatively stable, there are various intrinsic and extrinsic factors that can affect the composition of the GI microbiota throughout life[281], as described below.

2.2.1 Mode of delivery at birth

During the birth process and immediately after birth, the initial colonisation of the infant occurs from the mother and surrounding environment [282]. Thus, the mode of delivery (vaginally or by caesarean section) plays a pivotal role in the initial microbiota composition. Infants born pre-termly or by caesarean have been shown to have altered intestinal microbial colonisation beginning at 1 day which can persist to 7 years of age[283]. In contrast, vaginally delivered infants have a higher microbial diversity with the presence of gut bacteria such as *Lactobacilli*, *Bifidobacteria, Prevotella* and *Bacteroides* [284], due to a longer birth process which is associated with the presence of viable microorganisms in the GI tract of the infant[285]. These gut microbes are shared between the GI tract of infants and mothers' faeces immediately after birth, which suggests that the proximity of the birth canal and the anus play a crucial role in the transmission of microbes from the mother to the infant[286].

2.2.2 Drugs

Increasing evidence suggests that the use of drugs have a profound effect on the gut microbiota[287]. Likewise, microbes that are found in the gut also affects the efficacy of drugs[288, 289]. Broad-spectrum

antibiotics have been shown to reduce the overall bacterial diversity of the gut microbiota, which in some cases may lead to a high abundance of Proteobacteria and low abundance of Actinobacteria populations[290, 291]. Drug-related microbial dysbiosis of the GI tract can increase the abundance of commensal bacteria and increase colonisation of pathogens such as *Clostridium difficile*[292] or *Salmonella*[293]. Owing to the close links between the resident microbiota and the host, such disturbance of the microbiota by antibiotics can increase the risk of developing disorders such as obesity, asthma, eczema and inflammatory bowel disease[294, 295]. A recent study has reported that non-antibiotic drugs can also change the composition of the gut microbiota[296]. In this study, screening of more than 1,000 marketed drugs against 40 representative gut bacterial strains showed that 24% of human-targeted drugs, including members of all therapeutic classes, inhibited the growth of at least one strain *in vitro*[296]. In addition, drug-induced shifts in commensal microbes can lead to indirect but important changes in the immune response[297].

2.2.3 Diet

A dynamic equilibrium exists between the gut microbiota, host physiology, and diet that affects the initial colonisation, developmental succession, and eventual stability of the gut ecosystem. Numerous studies in humans and mice have demonstrated that diet is one of the key contributing factors shaping the gut microbiota composition and diversity[298, 299]. The GI tract of infants favours the colonisation of facultative anaerobic bacteria and genes involved in breakdown of polysaccharides such as lactose since their diet is enriched in milk[278, 300]. In addition, breast milk is known to contain short oligosaccharides that act as selective prebiotics to promote colonisation of the gut with beneficial bacteria that confer some protective effects against pathogens[301-303]. Furthermore, evidence exists for an entero-mammary pathway that transfers various bacterial species from the mother to the infant during breastfeeding[304, 305]. Many studies suggest that the gut microbial profile of breastfed infants is dominated by *Bifidobacterium*[306-310], with the addition of a few other anaerobes and small numbers of facultative anaerobic bacteria[310]. Diet continues to play a primary role in generating compositional change and diversity in the microbiome as dietary patterns progress over the first three years[311, 312]. Major shifts in the taxonomic groups of the microbiome have been observed with changes in diet such as weaning to solid[312]. The introduction of solid food to the breastfed infant causes a rapid rise in the number of enterobacteria and enterococci, followed by progressive colonization by *Bacteroides spp., Clostridium*, and anaerobic *Streptococcus*[311].

The composition of the healthy adult gut microbiota is relatively stable[274, 313-316]. However, it is very dynamic and responds to changes in environmental factors such as the diet. A common trend is that a diet rich in dietary fibres such as fruits or vegetables favours the colonisation and growth of *Bacteroidetes*, as fibres are a good source of microbiota accessible carbohydrates, which can be utilised by microbes to provide the host with energy and a carbon source[317, 318]. In contrast, consumption of high saturated and *trans*-fat diets promotes the growth of Firmicutes[319-321]. In addition, high-fat diets promote the growth of bacteria that are bile-acid tolerant, like *Alistipes* species and *Bilophila* species, as bile acids are bactericidal for many species[322]. Differentiation of fermentable substrate degradation potential contributes to metagenomic functional repertoire variation between individuals, with different enterotypes showing distinct saccharolytic/proteolytic/lipolytic profiles[323].

The long-term effects of a range of diets, including western, gluten-free, omnivore, vegetarian, vegan, and Mediterranean, have been studied for their ability to modulate the gut microbiota. In several studies, a Western diet (high in animal protein and fat, low in fibre) led to a marked decrease in numbers of total bacteria and beneficial *Bifidobacterium* and *Eubacterium species*[313, 324-326]. There is growing evidence that aspects of a 'Western diet' increase the risk of developing inflammatory bowel disease (IBD). More recently, evidence implicating dietary emulsifiers has accumulated, with ecological studies showing a positive correlation between IBD and emulsifier consumption[327] (for a review, see[328]). Recently, a deep metagenomic sequencing of 1,203 gut microbiomes from 1,098 individuals enrolled in the Personalised Responses to Dietary Composition Trial (PREDICT 1) study found many significant associations between microbes and specific nutrients, foods, food groups and general dietary indices, which were driven especially by the presence and diversity of healthy and plant-based foods[329].

2.2.4 Host genetics

Although environmental factors have a substantially strong effect on microbiome composition, the genetic makeup of the host is also believed to play a role in shaping the overall microbiome composition[330]. The gut microbiota diversity is more similar between family members than between unrelated individuals that have similar diet, and the gut microbiota is more similar in monozygotic than between dizygotic twins. Several lines of evidence point towards a possible coevolution of the resident gut microbiota with the host as demonstrated by faecal microbiota transplantation which resulted in the morphing of the transplanted populations to resemble the indigenous microbiota of the recipient host[331-333]. Intestinal mucin glycosylation, host-derived antimicrobial proteins, immune mediators, lectins, or bile acid can directly influence the composition of the gut microbiota[334]. The effect of host

genetics on gut microbiome composition was recently analysed in detail by the MiBioGen consortium which curated and analysed genome-wide genotypes and faecal microbiome sequencing data from 18,340 individuals (24 cohorts)[335].

2.3 The physiological roles of the gut microbiota

The intestinal microbiota confers numerous health benefits to the host, as discussed below.

2.3.1 Carbohydrate fermentation

Symbiotic bacteria metabolise otherwise indigestible polysaccharides, supplying essential nutrients and maintaining energy homeostasis. Incompletely fermented fibres such as insoluble cellulose, promote and maintain intestinal health through increasing digesta mass which dilutes toxins, reduces intracolonic pressure and shortens transit time[336, 337]. *Bacteroides* species are particularly well equipped at degrading complex dietary fibres such as xylan and its oligosaccharides xylooligosaccharides and arabinoxylooligosaccharides[338], or pectin[339], one of the most complex polysaccharides on earth, due to the wide repertoire of carbohydrate-active enzymes they produce[340]. The fermentation of polysaccharides by the gut microbiota results in the production of short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate[341 342, 343]. SCFAs such as butyrate are quickly absorbed in the colon which serve as energy source to the colonocytes[343]. Acetate which is the most abundant SCFA not only in the gut lumen but also in peripheral circulation[344], and can mediate fat accumulation via the GPR43 signalling pathway[345]and/or affecting appetite via a central homoeostatic mechanism[346]. Propionate is transferred to the liver to be used as a substrate for gluconeogenesis and exert several physiological functions[347, 348].

2.3.2 Protection against pathogens

The physical presence of the commensal bacteria in the GI tract contributes to the protection of the host from pathogens through a mechanism known as competitive exclusion by competing with pathogens for nutrients and attachment sites[349]. The gut microbiota, through its structural components and metabolites, can also stimulates the host to produce an array of antimicrobial compounds such as cathelicidins, C-type lectins and (pro)defensins by the host Paneth cells via a PRR-mediated mechanism[350]. Another mechanism by which the gut microbiota can limit pathogen overgrowth is by inducing the production of mucosal SIgA[351]. SIgAs are then anchored in the outer layer of colonic mucus through combined interactions with mucins and gut bacteria, thus providing immune protection against pathogens whilst maintaining a mutually beneficial relationship with commensals[352] (**see section 1.3.1**).

Moreover, a recent study has showed that acetate—one of the major gut microbial metabolites—not only increases the production of SIgA in the colon, but also alters the ability of the SIgA to bind to specific microorganisms such as *E. coli*[353].

2.3.3 Modulation of the immune system

The gut microbiota is also required for the development and maturation of the immune system[354]. The study of causal relationships between gut bacteria and host immunity is strongly informed by the use of GF mice models[355]. GF mice display numerous malfunctions, including an extensive defect in the development of antibody[356, 357], reflecting an immature immune system [358-362], which can be reverted by the addition of the gut microbiota. For instance, increased expression of TGFβ and IL-10, which are known for their anti-inflammatory activity, was observed after administration of a cocktail of commensal bacteria to GF mice[363]. Furthermore, IgA antibodies, which are the mainstay of protective humoral mucosal immunity, show substantial reduction in GF animals, and can be strongly induced upon de novo colonisation[364]. The LP found in the small intestine contains a large number of IL-17^{+}CD4^{+} T (Th17) cells, which represent a class of potent immunomodulatory effector cells[365]. In GF mice, Th17 cells are absent in and are inducible upon microbial colonisation, most notably with segmented filamentous bacteria[365, 366], but also other commensal bacteria such as *Bifidobacterium adolescentis*[367]. Some other commensal bacteria such as *L. rhamnosus GG* and *L. acidophilus*, have also been shown to downregulate the expression of Th17 cells and secretion of IL23 and IL17 via inhibition of STAT3 and NF-κB signalling DSS-induced mouse models of ulcerative colitis[368, 369]. humans, insufficient microbial exposure during birth or the early life can lead to altered gut microbiota composition and defective or allergy-susceptible immune system in adults[370].

3. *LACTOBACILLUS REUTERI*: A MODEL ORGANISM TO STUDY HOST ADAPTATION

3.1 Occurrence in the GI tract

Bacteria traditionally classified in the genus *Lactobacillus* comprised over 250 species and 29 subspecies of a paraphyletic group of Gram-positive, facultatively anaerobic, rod-shaped, non-spore-forming bacteria. Recently, based on whole genome sequences, the genus *Lactobacillus* has been reclassified into 25 genera, which includes 23 novel genera[371]. This reclassification reflects the phylogenetic position of the microbes and categorises *lactobacilli* into clades that share ecological and metabolic properties. Together with functional analyses of representative species of the *Lactobacillus* genus, these genomic, metabolic and phylogenetic metadata suggest a high level of niche conservatism within the well-supported phylogenetic groups within the genus, with lifestyles ranging from non-symbiotic (free-living)

to strictly symbiotic[372]. The findings support a model in which host-adapted *Lactobacillus* lineages evolved from non-symbiotic ancestors, with recent species displaying considerable dissimilarity in the degree of host specificity and their reliance on environmental niches. This genus is considered one of the main genera in the GI tract of several vertebrates, and lactobacilli are found in variable amounts depending on the species, their location within the gut and age of host[278] and due to their presence in breast milk, are one of the first bacteria to colonise the mammalian GI tract[373]. The bacterial species *Lactobacillus reuteri*, recently renamed *Limosilactobacillus reuteri*[371] according to the new classification, inhabits the GI tract of a variety of vertebrates (for a review see[374]).

L. reuteri is a heterofermentative bacterial species that grows in oxygen-limited atmosphere in a variety of ecological niches including the human gut, as well as in the GI tract of other vertebrates, including rodents, pigs and chicken[375]. A series of phylogenetic, phylogenomic, and experimental mouse studies have established this species as a paradigm for host adaptation (**Figure 17**)[376]. Comparative genetic hybridisation studies using 57 *L. reuteri* strains from six different hosts showed fundamentally different trends of genome evolution in different hosts. For example, rodent isolates although showing a high degree of genomic plasticity, possessed some specific genomic features that were rare or absent in strains from other vertebrate hosts[377].

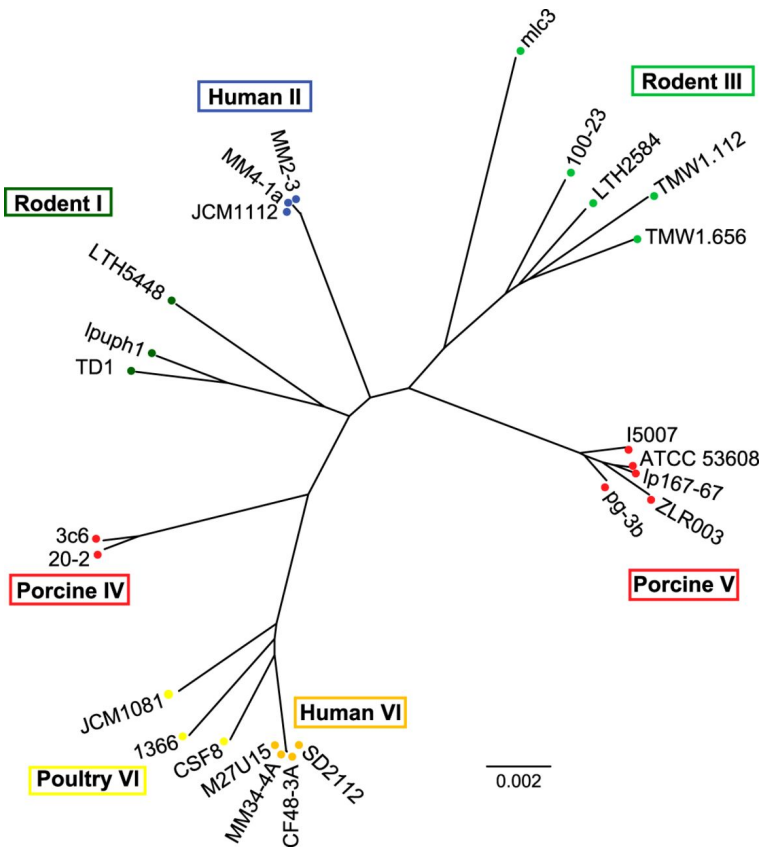

Figure 17*:* Neighbour-joining phylogenetic tree of *L. reuteri* based on the core genome alignment (900 genes) of 25 strains.

Tips of the branches are colour coded by lineage, and cohesive clades are labelled**.** Taken from Duar et. al., 2017.

The unique genome content of *L. reuteri* lineages reflects the niche characteristics in the gut of their respective hosts. Rodent isolated strains have been shown to display elevated fitness in mice[31, 376], and biofilm formation in the forestomach is restricted to strains from rodent lineages[378]. Inactivation of the major representative rodent-specific genes in *L. reuteri* resulted in an impaired ecological performance in the murine[378] (**Figure 18**). Host adaptation of *L. reuteri* strains to both rodents and chickens was recently demonstrated experimentally by administrating standardised inocula composed of *L. reuteri* strains from different host-confined lineages to mice, pigs, chickens, and human. Together these findings suggest that *L. reuteri* has evolved via natural selection in several vertebrate hosts[372] .

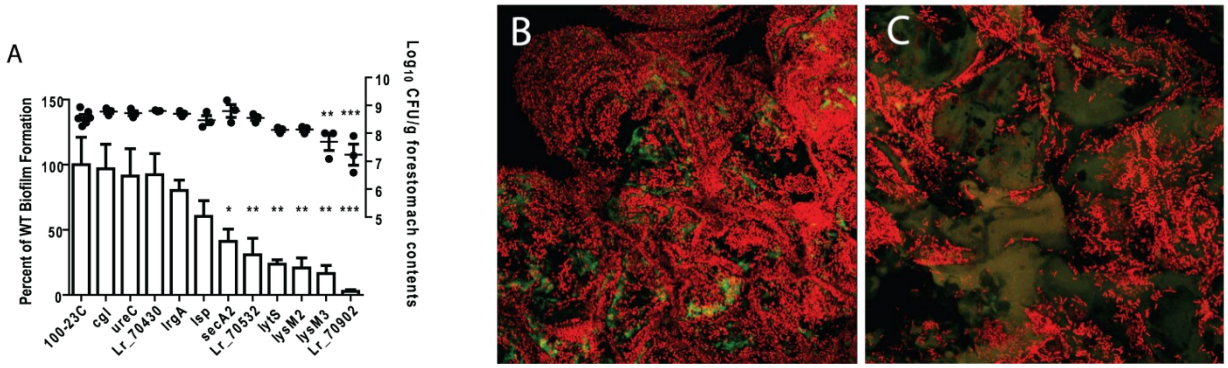

Figure 18: Characterisation of in vivo biofilms of mutant strains of *L. reuteri* 100-23.

Germ-free mice were colonised for two days after gavage with single dose of ~10^7 cells of mutant strains or wild-type 100-23C. Biofilm density was compared by confocal microscopy of forestomach tissue. **(A)** Quantification of biofilm density (relative to biofilm of wild-type 100-23C. **(B)** Micrograph of wild type, **(C)** Micrograph of secA2 mutant. Adapted from Frese et. al., 2013.

4.2 Molecular determinants of host adaptation

L. reuteri is a large component of biofilms that line parts of the upper digestive tract of mice, rats, pigs and chicken[379-382], whereas it is considered one of the true autochthonous species of the human GI tract[383]. Several colonisation factors such as reuterin[384, 385], lactic acid[386], exopolysaccharides (EPS)[387] or cell surface proteins involved in biofilm formation or binding to the mucus or the epithelium have been functionally characterised in *L. reuteri* strains[388].

4.2.1 L. reuteri exopolysaccharides (EPS)

EPS are glycopolymers present on the surface of Gram-positive and Gram-negative bacteria. In terms of structure, two main groups of EPS are produced by bacteria: heteropolysaccharides (HePS) and homopolysaccharides (HoPS)[389]. HePS are comprised of two to eight repeating units of monosaccharides which are assembled by cell wall-bound glycosyltransferases in low quantities from intracellular sugar nucleotide precursors[390] whereas extracellular glycansucrases (glucan- or fructansucrases) synthesise HoPS consisting of either glucose or fructose from sucrose[391, 392]. EPS formation by glycansucrases has been reported for *Lactobacilli* of the species *L. reuteri*, *L. pontis*, *L. panis*, *L. acidophilus*, and *L. frumenti*[393]. HoPS synthesis and the corresponding genes have been characterised in the cereal associated *L. reuteri* TMW 1.106 and TMW 1.974 strains with homologues found in the accessory genomes of pig isolated strains (**Figure 19**)[394]. A cluster of more than 25 genes, including several encoding Gtfs, that may contribute to EPS synthesis have also been described in other *Lactobacillus* strains, including *L. rhamnosus* GG[395], was also reported in the *L. reuteri* human strain ATCC 55730[396].

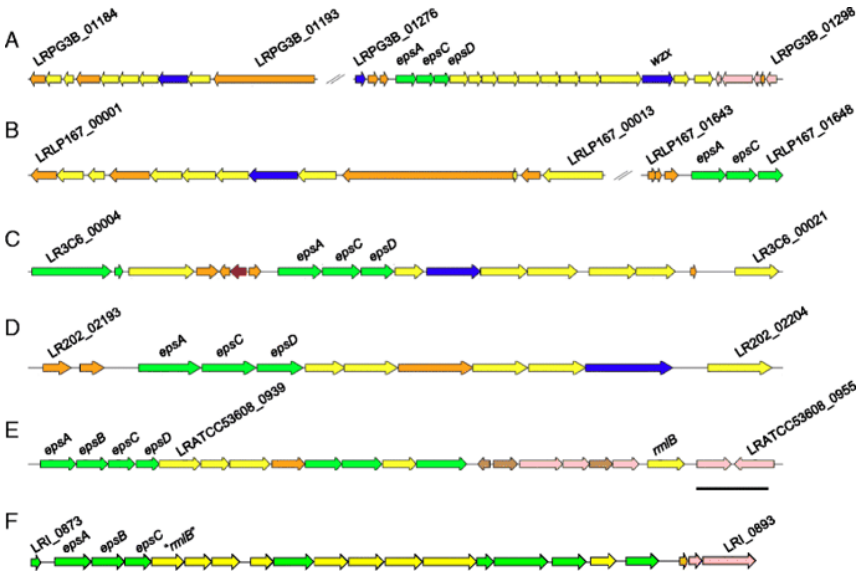

Figure 19: Predicted EPS gene clusters in *L. reuteri* pig strains.

(A) pg-3b, **(B)** lp167-67, (**C)** 3c6, **(D)** 20-2, **(E)** ATCC 53608 and (**F)** I5007. Colour scheme: yellow = Gtf; green = other protein involved in polysaccharide biosynthesis; blue = membrane protein; orange = hypothetical protein; pink = transposase/mobile element protein; brown, pseudogene; red = RNA polymerase σ subunit. Taken from Wegmann et. al., 2015.

HoPS biosynthesis of different *L. reuteri* strains has been previously investigated in detail (**Table 1**). *L. reuteri* strains often produce glucans and fructans of different linkage types, and some glycosyltransferases responsible for their production such as glucosyltransferase A (*gtfA*)[397], Fructosyltransferase (*ftf*)[398], *gtfML1*[399] and dextransucrase[399] have been biochemically characterised.

Table 2: EPS producing *L. reuteri* strains

Bacteria	Origin	HoPS monosaccharide component	EPS	Reference
L. reuteri TMW1.656	Sourdough	Glucose	Reuteran	[400]
L. reuteri LTH5794	Human intestine	Fructose	Fructan	[400]
L. reuteri FUA3048	Mouse intestine	Glucose	Glucan	[401]
L. reuteri 121	Sourdough	Glucose and Fructose	Glucan	[397]
L. reuteri LTH5448	Sourdough	Fructose	Fructan	[402]
L. reuteri 1.693	Human intestine	Fructose	Fructan	[393]
L. reuteri 1.649	Sourdough	Fructose	Fructan	[393]
L. reuteri 1.977	Duck colon	Glucose	Glucan	[403]
L. reuteri 1.106	Sourdough	Glucose	Glucan	[393]
L. reuteri 100-23	Mouse forestomach	Fructose	Fructan	[387]

EPS have been implicated in protection against toxic compounds, bacteriophages, osmotic stress, bacterial desiccation, and adhesion to solid surfaces and biofilm formation[404]. The EPS produced by *L. reueri* TMW1.656 was shown to be important for biofilm formation and inhibited Enterotoxigenic *E. coli* (ETC)-induced hemagglutination of porcine erythrocytes[401]. Furthermore, difference in the chemical structures and immunomodulatory properties has been shown in EPS found on lactobacilli isolated from mice with IBD compared to healthy mice[405]. EPS biosynthesis has also been implicated in *in vivo* biofilm formation of *L. reuteri 100-23*. The production of EPS was abolished by mutating the ftf gene in *L. reuteri* 100-23 strain. The colonisation of the *ftf* mutant in the forestomach and caecum of *Lactobacillus*-free mice was significantly reduced as compared to the wild-type strain[387], suggesting that EPS production improves the colonisation of strain 100-23 in the gut.

4.2.2 Bacterial membrane vesicles (BEVs)

BEVs have been recognised as a form of cell-cell communication used by almost all domains of life: bacteria, archaea and eukaryotes[406]. BEVs enclose several bioactive compounds of the parental bacteria such as proteins, nucleic acids, lipids and polysaccharides[407]. These molecules implicated in many pathological and physiological functions including nutrient acquisition, biofilm formation, stress response, delivery of toxins and virulence factors and invasion of host and immune regulation[408]. Most

knowledge comes from studies of outer membrane vesicles (OMVs) which are released in large amount by Gram-negative bacteria while the release of BEVs by Gram-positive bacteria may be limited due to the presence of a thick cell wall[406]. Recently, BEVs have been isolated from *L. reuteri* BBC3, a gut-associated commensal bacterium of Black-Bone chicken and shown to important in the maintenance of GI homeostasis against LPS-induced inflammatory responses[409]. However, the mechanism whereby *L. reuteri*-derived BEVs modulate these functions has yet to be fully determined.

4.2.2 L. reuteri cell surface proteins

Numerous studies have shown that *L. reuteri* can bind to components of the epithelium or the mucus layer which allows this species to colonise the GI tract of a wide range of vertebrates [410, 411]. A possible mechanism for the successful establishment by commensal bacteria in the gut is through specific surface structures proteins known as adhesins. In addition to the structurally characterised mucus binding proteins, multifunctional proteins have been implicated in the adhesion of *L. reuteri* to the GI epithelium as described below.

4.2.2.1 Moonlighting proteins

Several moonlighting proteins, structures that perform two or more functions in addition to their primary (originally identified) have been identified in *lactobacilli*[33, 412, 413]. These proteins do not have a typical conserved cell-surface-anchoring motif, and their cell-surface localisation is affected by environmental conditions. Today, more than 100 cytoplasmic proteins, mainly metabolic enzymes and molecular chaperones, have been identified in *L. reuteri* strains as moonlighting proteins with activity of adhesion or modulation of cell signalling processes[414]. These *L. reuteri* proteins include: the elongation factor Tu (EF-Tu)[415-417], glutamine synthetase and glucose-6-phosphate isomerase[418], glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[419, 420], the chaperonin GroEL[421], enolase [422]and the ATP-binding cassette (ABC) transporter[423, 424]. Recently, proteins related to ABC transporters (L. reu_0517, Lreu_0098, and Lreu_0296) and LPxTG anchor domain proteins involved in adherence and colonisation of *L. reuteri* were shown to be upregulated in the cell surface after GI fluid treatment[425].

4.2.2.2 Mucus binding proteins (MUBs)

MUBs have been identified as one class of surface adhesins involved in mechanisms of adherence of lactobacilli to the protective mucus layer covering the epithelial cells of the GI tract[411]. MUB exhibits the typical organisation of cell surface proteins from Gram-positive bacteria : a C- terminal LPXTG anchoring motif, a N-terminal signal peptide and variable numbers of mucus binding domains, Mubs[426]. Proteins containing one or more repeats of the Mub domain are often found in lactobacilli that colonise the gut,

suggesting that Mub domains are functional units that may be the result of an evolutionary adaptation for survival in the gut[427]. The considerable diversity of MUBs among *L. reuteri* strains and the variation in the abundance of cell-surface MUBs significantly correlates with their mucus binding ability[411]. The strain-specific role of MUBs in recognising mucus elements and/or their capability of promoting aggregation can explain the contribution of MUBs to the adherence of *L. reuteri*[388]. The best studied MUB protein to date is from *L. reuteri* ATCC 53608 (**Figure 20**) which comprises two types of amino acid repeats (Mub1 and Mub2); six copies (RI–RVI) of the type 1 repeat (Mub1) and eight copies (R1–R8) of the type 2 repeat (Mub2)[428]. X-ray crystallography revealed that each repeat is further divided into two domains, a mucin binding domain and an immunoglobulin binding (Ig-binding protein) domain[429]. Atomic force microscopy (AFM) suggested that Mub repeats may be involved in the interaction with mucin glycans, via binding with terminal sialic acid moieties[430].

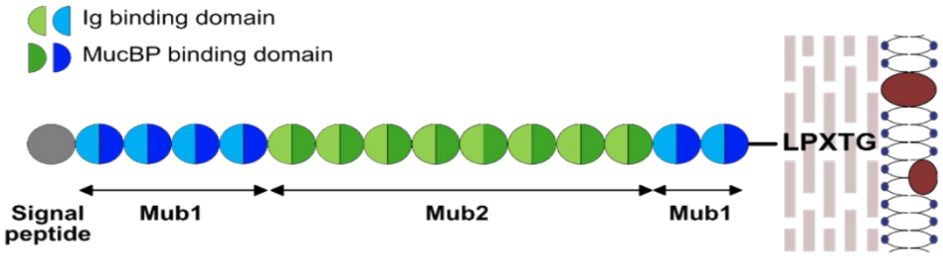

Figure 20: Schematic representation of MUB from *L. reuteri* ATCC 53608.

MUB comprises six Mub type 1 repeats (blue), eight Mub type 2 repeats (green). Each Mub repeat has two domains, an Ig binding domain at the N-terminus and a mucin binding domain (MucBP) at the C-terminus. The protein also has an N-terminal signal peptide domain (grey). The C-terminal LPXTG-motif (black) anchors MUB to peptidoglycan of the bacterial cell wall. Taken from Etzold and Juge, 2014.

This elongated structure allows for the exposure of all 14 repeats, each of which has the capacity to bind to mucus components[428]. This is proposed to allow the bacteria to inhabit the outer mucus layer, in contrast to many pathogenic adhesins which show adhesion to the epithelial surface via the N-terminal tip. Furthermore, MUB from *L. reuteri* ATCC 53608 has been shown to induce an inflammatory response *in vitro*[268]. In this study, MUB increased the capacity of *L. reuteri* strains to interact with monocyte derived DCs, promoted phagocytosis and increased the secretion of proinflammatory cytokines such as TNF-α, IL-1β and IL-6[431]. These immunomodulatory properties were shown to be mediated via interaction with innate C-type lectin receptors, Dectin-2 and DC-SIGN using cell reporter assay and AFM *in vitro*[268], suggesting that MUB may be glycosylated and have both adhesion and immunomodulatory properties. Furthermore, MUB from the *L. reuteri* ATCC 53608 strain was shown to increase bacterial binding to both mucus non-secreting HT-29 and mucus-secreting LS174T epithelial cells[432].

4.2.2.3 Cell and mucus-binding protein A (CmbA)

Molecular and structural approaches were used identify and characterise novel mucus-adhesins specific to *L. reuteri* human isolates[428]. Two studies reported the identification of a novel cell-surface protein, CmbA from *L. reuteri* ATCC PTA 6475, which is involved in the adhesion of *L. reuteri* human strains to cell and mucus[432, 433]. CmbA is a modular protein of 133 kDa containing six repeat domains, an N-terminal signal sequence and a C-terminal anchoring motif (LPXTG). The crystal structural of CmbA repeat showed a divergent immunoglobulin (Ig)-like β-sandwich fold, sharing structural homology with the Ig-like inter-repeat domain of internalins of the food borne pathogen *Listeria monocytogenes* which implicate the protein in adhesion of the bacteria to the intestine[434]. The involvement of CmbA in adhesion to mucus was demonstrated using inhibition assays with an anti-CmbA antibody and using a *L. reuteri* ATCC PTA 6475 CmbA KO mutant showing a significant reduction in binding to mucus *in vitro*[434]. In addition, overexpression of CmbA in strain ATCC PTA 6475 resulted in an increased adhesion to Caco-2 cells and mucus compared with the WT strain, indicating a dual specificity to intestinal cell and mucus[433].

4.2.2.4 Serine rich repeat proteins (SRRPs)

SRRPs are a family of large, surface-exposed proteins that are found in Gram-positive bacteria. These proteins have been mostly studied in pathogenic bacteria such as *Staphylococcus* and *Streptococcus pneumoniae*[435]. However, recently SRRPs have been identified in *L. reuteri* strains[378,165]. SRRPs form large stalks or fimbriae-like structures that extend outward from the bacterial surface to mediate adhesions[436]. SRRPs are organised into several domains which include an unusually long N- terminal

signal peptide, followed by alanine-serine-threonine rich region, a short serine-rich domain (SRR1), a binding region (BR), a second much bigger serine-repeat-rich region (SRR2), and a C- terminal LPXTG cell wall anchoring motif[437] (**Figure 21**). The crystal structures of the binding regions (BRs) of $SRRP_{100\text{-}23}$ and $SRRP_{53608}$ from *L. reuteri* 100-23 and ATCC 53608 strains, revealed a unique β-solenoid fold in this important adhesin family. $SRRP_{53608}$-BR bound to host epithelial cells and DNA at neutral pH[165].

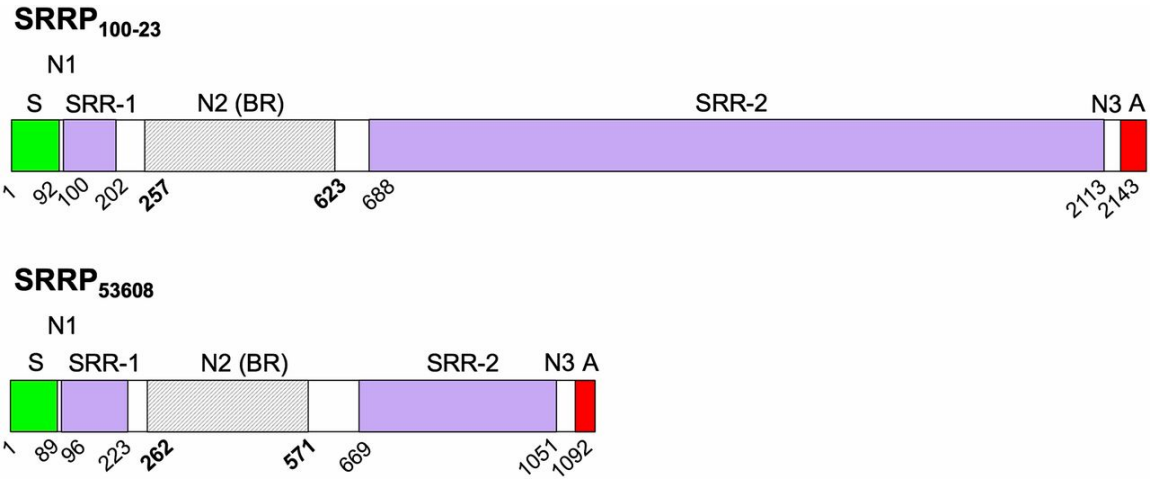

Figure 21: Schematic representation of SRRP from *L. reuteri* 100-23 and ATCC 53608.

The two proteins are drawn to scale. **(A)** cell wall anchor including LPXTG motif; **(N1)**, nonrepeat region 1; **(N2) (BR)**, nonrepeat region 2; **(N3)** nonrepeat region 3; S, secretion signal sequence; SRR-1, serine-rich region 1; SRR-2, serine-rich region 2. The beginning amino acid position is indicated below each domain. Regions of the BR that were resolved by crystallography are shaded gray and span amino acids 257–623 for SRRP100-23 and amino acids 262–571 for SRRP53608. Taken from Sequeira et. al., 2017

Both SRR1 and SRR2 of SRRPs are usually subject to heavy glycosylation, which is important for the stability and transport of nascent SRRPs in cytoplasm[438]. Transport of SRRPs onto the bacterial surface occurs through a dedicated non-canonical Sec translocase, Sec-Y2A2, following recognition of an extended atypical signal sequence peptide at the N-terminus [439]. The Sec-Y2A2 system consists of two translocases, SecA2 and SecY2, three accessory Sec system proteins (Asp1–3), and a variable number of glycotransferases (GTs). The core genetic component of these loci, found in all strains that carry an SRRP, includes two GT-encoding genes (most commonly called GtfA and GtfB) involved in the first step of SRRP glycosylation, and five genes encoding components of the alternate *secY2A2* complex necessary for SRRP transport [*secA2*, *secY2*, *Asp 1-3* (also known as *gap 1-3*); *Asp 1-3* may also be involved in glycosylation[440]. To date, the accessory secretion (aSec) system has been identified in the genomes of various murine and porcine *L. reuteri* strains, however no aSec secretion system appears to be present in human isolates[165, 378, 394, 441]. The accessory Sec cluster is conserved in *L. reuteri* ATCC 53608 and 100-23[394] strains but with variations in the genomic organisation and number of glycosyltransferases, suggesting strain-specific differences in glycosylation of *L. reuteri* SRRPs (**Figure 22**).

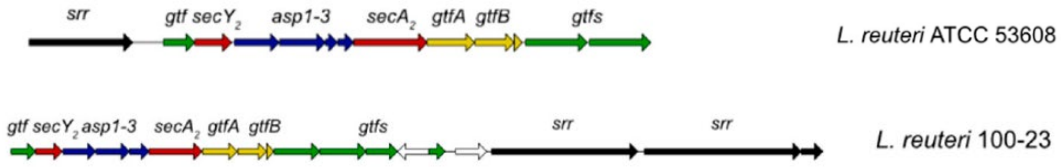

Figure 22: Organisation of the secA2 clusters identified in *L. reuteri* 100-23 and *L. reuteri* ATCC53608.

The genes encoding the translocases $SecA_2$ and $SecY_2$ are shown in red, the accessory secretion proteins asp1–3 in blue and the priming GTs, GtfA and GtfB, in yellow. Genes encoding additional GTs are shown in green and the genes encoding serine-rich repeat proteins are illustrated in black. Taken from Latousakis and Juge, 2018.

The glycosylation profile of SRRPs from *L. reuteri* strains was recently determined using a combination of bioinformatics analysis, lectin screening, LC-MS-based sugar nucleotide profiling, MALDI-ToF, and GC-MS analyses[442]. This study showed that the *L. reuteri* ATCC 53608 and 100-23 strains were capable of performing protein glycosylation and that $SRRP_{100\text{-}23}$ and $SRRP_{53608}$ were glycosylated with Hex-HexHexNAc and di-HexNAc moieties, respectively. Following *in vivo* glycoengineering in *E. coli*, NMR analysis and enzymatic treatment further showed that $SRRP_{53608}$ was glycosylated with GlcNAcβ(1→6)-GlcNAcα moieties. Together, it was suggested that $SRRP_{100\text{-}23}$ is glycosylated with GlcNAc and Hex-Glc-GlcNAc whereas $SRRP_{53608}$ is glycosylated with GlcNAc and di-GlcNAc moieties (**Figure 23**).

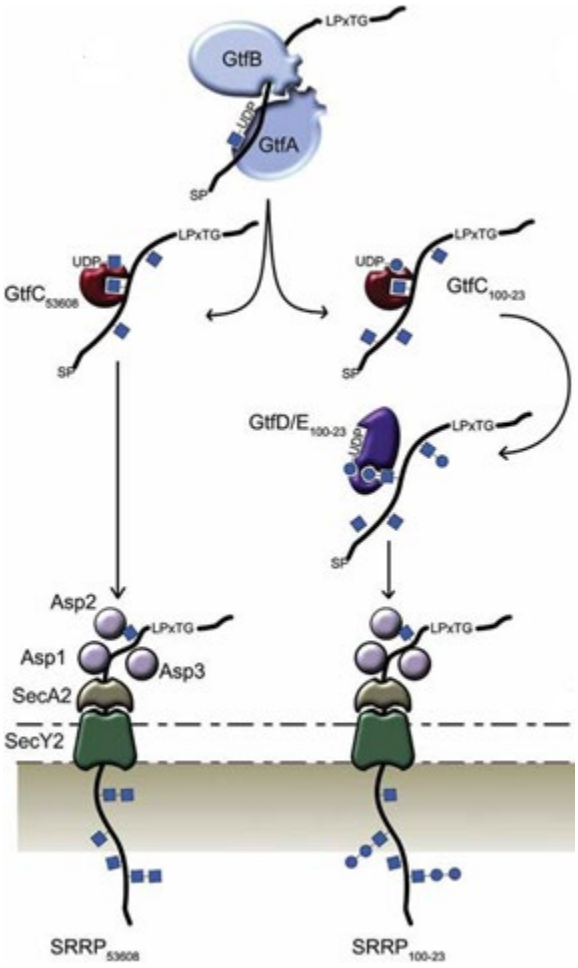

Figure 23: SRRP glycosylation mechanisms in *L. reuteri* strains.

SRRP glycosylation in *L. reuteri* strains ATCC 53608 and 100-23. The GtfA/B complex initiates the glycosylation of the *L. reuteri* SRRP with GlcNAc residues, while GtfC extends the glycans with either GlcNAc ($GtfC_{53608}$) or Glc ($GtfC_{100-23}$). The glycosylated $SRRP_{53608}$ is then secreted through the aSec system, whereas the $SRRP_{100-23}$ is further extended by GtfD and/or GtfE before secretion. After extension and acetylation of the glycans by other enzymes in the aSec system, the adhesins are secreted through the SecA2/Y2 channel. Blue circle: glucose, blue square: GlcNAc, SP: signal peptide. Taken from Latousakis et. al., 2019.

The aSec cluster and SRRP in the murine isolate *L. reuteri* 100-23 is crucial for adhesion of the bacteria to the forestomach epithelium of the murine GI tract. *L. reuteri* mutants lacking the secA2 gene showed defective adhesion, whereas mutants lacking the srrp gene showed the most reduced biofilm formation, compared to other targeted adhesins tested (**Figure 18)**[378]**.** Together, these studies suggest that the *L. reuteri* SRRPs are crucial for bacteria-host interactions and that glycosylation may contribute to biofilm formation and host specificity of *L. reuteri* strains.

4.3.1. Evidence for L. reuteri immunomodulatory properties

Numerous studies showed that *L. reuteri* strains can modulate the immune system. For example, in mice, *L. reuteri* strains have been shown to trigger a strain-specific activation cascades that either exert an immune response (pro-inflammatory effect) or attenuate the immune response (anti-inflammatory effect)[443, 444]. In addition, supplementation of *L. reuteri* DSM17938 in scurfy mice (characterised by a complete functional deficiency of Treg) mice, led to a complete functional deficiency of regulatory T cells, improved multiorgan inflammation induced by $Foxp3^+$ Treg deficiencies and raised survival rate[445]. In animal model of brain inflammation, *L. reuteri* DSM 17938 supplementation attenuated Th1/Th17 cells, restored gut microbial biodiversity, and reduced the development of experimental allergic encephalomyelitis [446].

L. reuteri has also been shown to enhance systemic TLR7 signals and promote plasmacytoid dendritic cell (pDC) recruitment to non-gut organs in a TLR7 overexpressing mice[447]. In addition, *L. reuteri* strains DSM 17938 and ATCC PTA 4659 differentially modulated inflammation in rats[448]. In this study, both strains significantly increased survival rate and decreased the incidence and severity of necrotizing enterocolitis. Treatment of the rats with these *L. reuteri* strains also downregulated mRNA expression of IL-6, TNF-α, TLR4, and NF-κB, while mRNA levels of anti-inflammatory cytokine IL-10 were significantly upregulated. The importance of TLRs in mediating the interaction between the immune system and *L. reuteri* strains was further highlighted in a study showing that the protective effect of *L. reuteri* DSM 17938 against experimental necrotizing enterocolitis is mediated by TLR2 in mice[449].

In another study, mice with colitis induced by immune checkpoint blockade showed a reduction in faecal *Lactobacillus*[450], and *L. reuteri* administration stopped the progression and development of colitis, likely through reducing the effects of immune checkpoint blockade on ILC3 expansion[450]. Similarly, *L. reuteri* has been shown to be protective in chemically DSS-induced colitis[451, 452] by preventing inflammatory platelet- and leukocyte-endothelial interactions[453]. A cocktail of *L. reuteri* strains (two isolated from rat, R2LC and JCM 5869, and two from human sources, ATCC PTA 4659 and ATCC 55730) has been shown to

improve DSS-induced murine colitis *in vivo* by increasing mucus thickness[454, 455]. In addition, meta-analysis of the effect of dietary probiotic supplementation has shown that *L. reuteri* bacteria were effective for colon length, TNF-α, IL-6, IL-10, IFN-γ, and disease activity index in murine IBD models[456]. In line with the homeostatic role of *L. reuteri* in the gut, supplementing mouse models of high salt-induced experimental autoimmune encephalitis improved disease through reduction in Th17 cells[457]. Furthermore, a cocktail of probiotic *Lactobacillus* strains has been shown to improve lupus-like disease in the MRL/lpr (mice models of systemic lupus erythematosus) although *Lactobacillus* strains, in particular *L. reuteri*, were shown to induce pathogenicity in other lupus mice models[319].

At the molecular level, *L. reuteri* EPS has Been implicated in anti-inflammatory properties. For example, *L. reuteri* EPS-mediated blocking of adhesion has been shown to suppress gene expression of pro-inflammatory cytokines that are induced by *E. coli* infection, including IL-1β and IL-6 intestinal epithelial cell lines *in vitro*[458]. This was confirmed *in vivo* as supplementation with EPS from *L. reuteri* prevented piglet diarrhoea in bacterial infection by reducing the adhesion of *E. coli*[459]. Using transcriptomics, it was possible to comprehensively map the biological processes in porcine intestinal epithelial cells challenged with EPS derived from *L. reuteri* alone, ETEC or ETEC after pre-treatment with EPS[458]. EPS produced by rodent *L. reuteri* 100-23 was also shown to induce Foxp3$^+$ Treg cells in the spleen[387]. Furthermore, recent work showed that the galactose content of EPS from *L. reuteri* Mh-001 could enhance anti-inflammatory effects on phagocytic immune cells such as macrophages[460].

In humans, evidence for the role of *L. reuteri* in promoting health has been evaluated through systematic reviews and meta-analyses of clinical trials[461-465], showing reduction in disease severity of several chronic illnesses such as infantile colic, chronic periodontitis, *Helicobacter pylori* infection and peri-implant diseases. but the underlying mechanisms supporting *L. reuteri* immunomodulatory properties in these studies are scarce **(Table 2)**. For example, anti-inflammatory properties through expansion of Treg cells after consuming a 'probiotic' yogurt containing certain *L. reuteri* and *L. rhamnosus* strains have been reported in patients suffering from IBD[466]. In another double-blind, placebo-controlled randomised study, reduction in crying time was observed in infants with colic following treatment with *L. reuteri* DSM17938, which was associated with upregulation of Treg and TLR2 and 4 mRNA expression[467].

Table 3: Summary of studies on the *in vivo* immunomodulatory effects of *L. reuteri* strains.

Strain	Subjects	Result	Reference
L. reuteri DSM 17648	Adults with *H. pylori infection*	Decrease in pathogen load in the stomach	[468]
L. reuteri DSM 17938	Adults with *H. pylori infection*	Successful eradication of the pathogen	[469]
L. reuteri DSM 17938	Adults with cystic fibrosis	Rescued gut microbiota dysbiosis	[469]
L. reuteri DSM 17938	Infants aged up to 6 months	*L. reuteri* supplementation significantly shortened the crying duration, but a causal relationship between the modulatory effect of probiotics on microbiota and the immune system has not been confirmed.	[465]
L. reuteri DSM 17938	Infants with colic	Significantly reduced crying time	[467]
L. reuteri DSM 17938	Children with acute gastroenteritis	Significantly reduced the duration of watery diarrhoea	[470]
L. reuteri DSM 17938	Infants with acute diarrhoea	Significant reductions in length of hospital stay, time to reach full feedings, and duration of hospitalisation, as well as nonsignificant reductions in the incidence of severe NEC and all-cause mortality	[461]
L. reuteri 55730	Infants with colic	Significantly reduced crying time	[471]
L. reuteri DSM 17938	Infants with chronic constipation	Significantly increased the frequency of bowel movement	[472]

L. reuteri ATCC PTA 6475	Adults with *H. pylori infection*	Reduced urease activity in pantoprazole therapy	[469]

CHAPTER 2 - HYPOTHESIS AND AIMS

This project aims to test the hypothesis that the ability of *L. reuteri* strains to colonise the gut and trigger immune response is mediated by protein-glycan interaction between the bacterial glycosylated adhesins and the host epithelium or immune system. Specific objectives include:

(i) Determining the molecular determinants involved in the interaction between CTLs expressed by immune cells and *L. reuteri* adhesins *in vitro*,

(ii) Investigating the role of cell-surface adhesins on the adaptation of *L. reuteri* strains to the gut.

(iii) Characterising the impact *of L. reuteri* adhesisns on the host immune response.

CHAPTER 3 - MATERIALS AND METHODS

3.1 Microbiology-based assays

3.1.1 Bacteria and culture conditions

The list of *L. reuteri* strains used in this study is provided in Table 3. *L. reuteri* strains were routinely cultured in de Man, Rogosa and Sharpe (MRS) culture medium (Oxoid, Ireland), which is selective for *Lactobacillus* at low pH (6.2 ± 0.2), or semi-defined substrate medium, *Lactobacillus* defined medium II (LDMII) (Kotarski and Savage, 1979). *L. reuteri* cultures were inoculated from frozen glycerol stocks and grown in an anaerobic cabinet (5% CO_2, 10% H_2 and 85% N_2, Don Whitley Scientific) static at 37°C overnight. Erythromycin (5 mg/ml) was added to the growth medium for the culture and maintenance of *L. reuteri* insertion mutants listed below. For cultures on plates, *L. reuteri* colonies were cultured at 37 °C on MRS agar (1.5% w/v agar, Formedium, UK) plates overnight under anaerobic conditions.

Table 4: Bacterial strains used in this study.

Strain	Host origin	Reference
L. reuteri ATCC 53608	Pig	Oh et. al., 2010
L. reuteri 1063N	Pig	MacKenzie et. al., 2010
L. reuteri 100.23C	Rat	Oh et. al., 2010
L. reuteri 100-23C ΔSRR	Rat	Frese et. al., 2013
L. reuteri 100-23C ΔGtfB	Rat	Frese et. al., 2013
L. reuteri 100-23C ΔAsp2	Rat	Frese et. al., 2013
L. reuteri 100-23C ΔSecA2	Rat	Frese et. al., 2013
L. reuteri 100-23C Δftf	Rat	Sims et. al., 2011
L. reuteri PTA 6475	Human	Kindly supplied by Jens Walter, University of Nebraska, Lincoln, USA
L. reuteri PTA 6475 cmbA KO	Human	Kindly supplied by Stefan Roos, University of Agriculture Science (SLU), Uppsala, Sweden.
Heat killed *Listeria monocytogenes (HKLM)*	Guinea pigs	Purchased from Invivogen (USA)
Escherichia coli (E. coli) BL21(DE3)	Human	Purchased from Merck (UK)

3.1.2 Microbial density and size characterisation

Bacterial density was quantified based on 600 nm (OD_{600nm}) of 1 corresponding to 8 x 10^8 cells/ml. Alternative methods for cell quantification included the use of the Bacteria Counting Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions, the use of flow cytometry (BD LSRFortessa), or imaging flow cytometry (Amnis ImageStreamx Mk II). For bacteria density and cell size quantification by ImageStreamx Mk II, bacteria at 10,000 events were collected and processed by IDEAS software. Bacteria density were found by selecting ''objects/ ml'' in the bright field channel (M04) of the Aspect Ratio_M04 versus Area_M04 dot plot. Bacteria cell size was determined by first selecting the low

intensity for side scatter laser (Channel 6) population (exclusion of control beads), and then applying the ''length'' feature in bright field.

3.1.3 Bacteria genome sequencing

3.1.*3*.1 Bacteria genomic DNA extraction

Chromosomal DNA of *L. reuteri* strains was isolated using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany). Bacterial (100 ng) DNA was further treated with 5 µL of DNase-free RNase (10 mg/ml) to remove RNA. Bacteria from 3 ml of an overnight culture (grown for around 16 h) were centrifuged at 10,000 x *g* for 10 min. Bacterial cell pellets were resuspended in 1 ml wash buffer (20 mM Tris-HCl, 2 mM sodium EDTA, pH 8.0) and centrifuged again using the same conditions. Genomic DNA was then extracted following the manufacturer's instructions for Gram-positive bacteria but with increasing the lysis incubation time at 37°C to 60 min. DNA was stored at -20°C.

3.1.3.2 Genome sequencing

The nucleotide sequences of the *L. reuteri* genomes were determined by whole-genome shotgun sequencing. The sample was run at a final concentration of 1.5 pM on an Illumina Nextseq500 instrument using a Mid Output Flowcell (NSQ® 500 Mid Output KT v2(300 CYS) Illumina). Data were uploaded to Basespace (www. basespace.illumina.com) where the raw data was converted to 8 FASTQ files.

3.1.3.3 Genome analysis

The Integrated Rapid Infectious Disease Analysis (IRIDA) Platform (irida.ca) was used to generate an assembled and annotated genome from reads using FLASH[473], SPAdes[474], and Prokka[475]. All predicted proteins were performed on the assembled *L. reuteri* genomes against different databases, including the nonredundant and the Refseq protein, databases provided by National Centre for Biotechnology Information (NCBI) and SWISS-PROT.

3.1.4 Exopolysaccharides (EPS)

3.1.4.1 Purification of EPS

EPS was isolated from *L. reuteri* strains using a modification of a published protocol (Horn et. al., 2013). Briefly, *L. reuteri* 100-23, *L. reuteri Asp2* mutant and *L. reuteri* ATCC53608 strains were grown in 10 l cultures in LDMII media, inoculated at 1% (v/v) with an overnight culture then incubated at 37°C for 2 days. Cells were harvested by centrifugation at 6,000 x *g* for 30 min at 4 °C and washed twice with phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The

washed bacterial pellet was resuspended in 300 ml 0.05 M EDTA to extract the EPS. The mixture was incubated under gentle agitation for 4 h at 4 °C then centrifuged at 6,000 x *g* for 30 min at 4°C. An equal vol f 100% ethanol was added to the supernatant to precipitate the isolated EPS from the bacterial cell pellet. Samples were centrifuged at 10,000 x *g* for 30 min at 4 °C and the pellet was resuspended in 50 ml of double-distilled water (ddH_2O) with gentle heating (50°C) for 2 hr. EPS was recovered by precipitation upon the addition of 2 vol of chilled ethanol. After centrifugation at 10,000 x *g* for 30 min at 4°C, the resulting EPS was resuspended in 50 ml ddH_2O with gentle heating (45°C) for 1 h followed by dialysis for 72 h (12,000–14,000-Da dialysis membrane) at 4°C, with two changes of ddH_2O per day. The material was freeze-dried and further purified by dissolving in 10% TCA and stirring overnight. The precipitated proteins were removed by centrifugation at 10,000 x *g* for 15 min at 4 °C. The pH of the supernatant was adjusted to 7 with 1 M NaOH and EPS was precipitated again with 2 volumes of chilled 100% ethanol. The pellet was dissolved in ddH_2O and then lyophilized by freeze drying. The purified EPS was stored at 4°C until further analysis. RNA, DNA and protein contaminations were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

3.1.4.1 Characterisation of purified EPS

A combination of 1D and 2D homo- and heteronuclear Nuclear Magnetic Resonance (NMR) (COSY, TOCSY, HSQC, HMBC, HSCQTOCSY) experiments were completed to establish the spin systems and therefore enable characterisation of individual polysaccharide structures. The spin system assignments of the extracted sample were recorded in D_2O at 300 K using a Bruker 600 MHz spectrometer equipped with a reverse cryo-probe. NMR sequences used for structural elucidation were: (^1H-^1H homonuclear) DQ-COSY (double quantum COSY spectrum), TOCSY, and NOESY, (^1H-^{13}C heteronuclear) HSQC, HMBC, and HSQC-TOCSY. The spectra were then processed and studied with Topspin 4.0 software (Bruker, USA).

3.1.5 Bacterial extracellular vesicles (BEVs)

3.1.5.1 Isolation of BEVs

L. reuteri derived BEVs were collected from cell culture supernatant as described previously by Liu et al. 2019 with some modifications. Briefly, *L. reuteri* cells were cultured in LDM II media in an anaerobic cabinet (5% CO_2, 10% H_2 and 85% N_2, Don Whitley Scientific) static at 37°C for 48 h. Cells were centrifuged at 6,000 x *g* for 30 min at 4°C. Supernatants were collected and vacuum filtered through a 0.22 μm membrane. The filtered supernatant was concentrated by spin-filtration at 5,500 x *g* and 4°C using a 100 K molecular weight cut-off filter unit (MWCO) (Sartorius, Germany). BEVs were recovered

from the filter using sterile PBS (2 ml) and further purified by density gradient ultra-centrifugation. For the gradient, Optiprep medium (60% w/v) was diluted in 0.85% w/v NaCl and 10 mM Tricine-NaOH pH 7.4 solution to make up 35%, 30%, 25% and 20% densities. In addition, BEVs in PBS were used to make a 40% Optiprep solution. BEVs were fractionated in a multiple-layered gradient in which the sample (in 40% w/v of Optiprep solution) was overlaid by 35%, 30%, 25%, 20%, 15% and 10% (w/v) using a 13.2 ml Ultra-clear tube (Beckman Coulter, UK). The tube was ultracentrifuged at 135,000 x *g* for 16 h at 4^{o}C with minimum acceleration and deceleration using a SW41 Ti rotor (Beckman Coulter, UK). From the top to the bottom, fractions (1 ml) were collected and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (**see section 2.2.5**). The BEV containing fractions (1 ml) were diluted 10 times (vol/vol) with sterile PBS and ultracentrifuged at 200,500 x *g* for 2 h at 4^{o}C using a Type 45 Ti rotor (Beckman Coulter, UK). BEVs were resuspended in sterile PBS (1 ml) filtered through a 0.22 μm membrane and stored at -80^{o}C.

3.1.5.2 Nanoparticle Tracking Analysis (NTA) of BEVs

Purified BEVs were quantified and measured in terms of particle size using the NanoSight LM12 (Malvern Panalytical). Briefly, BEVs were diluted 100-fold in ddH_2O, and the diluted samples were loaded into a syringe. The syringe was adjusted to the instrument's chamber and the sample was slowly released. The particle size of each BEV sample corresponded to the mean of triplicates. Instrument settings used were camera shutter 1035, camera gain 680, capture duration 60 s. The NTA software (version 3.0, NanoSight) was used to analyse BEVs samples, optimised to first identify and then track each particle on a frame-by-frame basis, and its Brownian movement is tracked and measured from frame to frame. The displacement co-efficient from the mean squared displacement of the particle tracked is calculated and substituting this value into the Stokes–Einstein equation allowed calculation of particle size. From each video, the mean, mode, and median BEVs size was used to calculate samples concentration expressed in nanoparticles/ml.

3.1.5.3 Transmission electron microscopy (TEM)

TEM analysis of *L. reuteri*-derived BEVs was performed by Dr Kathryn Gotts at the Quadram Institute Bioscience (Norwich) as follows. A 10 μl drop of BEVs suspension was placed onto a formvar/carbon coated copper TEM grid, left for 1 min, and then the excess liquid was wiped off. Immediately after, a 10 μl drop of 1.5% uranyl acetate solution was placed onto the grid and left for 1 min. The excess stain was removed using filter papers and the grids were left to dry completely before imaging. The grids were analysed in a FEI Talos F200C transmission electron microscope at 200kV.

3.1.6 Simulated gastrointestinal conditions

The simulated gastric and intestinal fluids solutions were prepared as described by Grimoud et al. (2010). Briefly, 1 ml of simulated gastric juice (NaCl 125 mM, KCl 7 mM, NaHCO3 45 mM, pepsin 3 g/L) at a final pH adjusted to 2.5 was inoculated with an overnight bacteria culture grown in LDM II media. Suspensions were incubated at 37°C. After 90 min incubation, bacteria were washed with PBS (pH 7.0) and the pellets were suspended in 1 ml of simulated intestinal fluid (pancreatin 0.1% w/v, bovine bile salts 0.15% w/v) at a final pH adjusted to 8.0 for 3 h under the same conditions. Bacterial viability was assessed by plating samples collected after incubation in simulated gastric fluid and after incubation in simulated intestinal fluid in LDM II media.

3.1.7 Carboxyfluorescein (cF) labelling of bacteria

For cF-labelling of the bacteria, *L. reuteri* strains were grown to early stationary phase in MRS or LDMII, cells were harvested following centrifugation (15,000 x*g*, 5 min, 4°C), washed twice in 0.01 M PBS containing NaCl (0.138M); KCl (0.0027 M) and resuspended in PBS (pH 7.4). Cell suspensions were adjusted to OD_{600nm} of 1 using a spectrophotometer. The bacterial cells (1 ml) were then labelled by incubation with 10 µM carboxyfluorescein diacetate (cFDA) (Merck, USA) at 37°C for 45 min. The cF-labelled cells were then centrifuged (15000 x g for 5 min at 4°C), washed, resuspended in PBS and used immediately.

3.1.8 Bacterial adhesion assays

3.1.8.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assay the binding between *L. reuteri* strains and mucin or galectin 3 (Gal-3). Purified porcine gastric mucin (pPGM, 100 µg) (100 ul) or 100 ul of purified recombinant Gal-3 (50 µg) was loaded onto high binding, black, polystyrene microtitre plate wells (Greiner Bio-One Ltd., UK) and incubated for 16 h at 4°C. The wells were washed three times with 200 µl of PBST buffer (PBS containing 0.05% (v/v) Tween 20). Then, 200 µl of blocking buffer (1% BSA in PBS) was added to each well and the plates were incubated at room temperature (RT) for 1 h. A total of 8 x 10^8 bacteria grown to early stationary phase (OD = 1) in LDM II medium and cF-labelled as above (**section 2.1.7**) were added to each well and incubated at 37°C for 1 h. The wells were washed again, and adhered bacteria were lysed by incubation at 37°C for 1 h with 200 µl of 1 % (w/v) SDS in 0.1 M NaOH. The released cF-fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) at 485nm and 520 nm as excitation and emission wavelengths, respectively. Negative controls of BSA coated wells were included by using 100 µg of BSA in PBS (100 ul) instead of pPGM or Gal-3 following the above process. Each assay

was performed in triplicate. Standards were included in triplicate consisting of 200 µl of SDS-lysed cF-labelled bacteria to evaluate the percentage of adhesion.

3.1.8.2 Biofilm assays

The biofilm capacity of *L. reuteri* strains to mucin was assessed on a Bioflux 200 system (Fluxion Biosciences, USA). Briefly, 2 microfluidic channels were primed with LDM II media (1 ml) for 30 min with no flow conditions. One channel was then coated with pPGM (0.5 mg/ml) for 1 h using the same conditions while the second remained uncoated. A total of 2 x 10^8 bacteria grown to exponential phase (OD = 0.25) in LDM II medium were then introduced in and allowed to attach to the surface for 30 min in the absence of flow. Planktonic and weakly adhered cells were then flushed from the test section by applying a flow rate at 0.3 dyn (~40 µl/h) and the cells were inspected under an inverted microscope (Axiovert 200M, Zeiss) for the formation of biofilm at 24 and 48 h. All microfluidic experiments were conducted at 37°C. Biofilm images were captured employing ZEN 2012 software (Zeiss) as combined tile images consisting of 81 one μm^2 horizontal tiles covering the entire microchannel. Actual growth quantification was recorded as integrated density on the entire combined tile image using Image J freeware (http://imagej.nih.gov/) using "integrated density", "mean value" and "area" as measurement settings.

3.1.8.3 Flow cytometry binding assays

Flow cytometry was used to assay the binding between *L. reuteri* strains and recombinant lectins, Dectin-2 and Gal-3. A total of 8 x 10^8 bacteria, grown in LDM II for 16 h and washed twice with sterile PBS was resuspended in 1 ml of PBS. For binding assays, bacteria (100 ul) were incubated for 1 h at 37˚C with 100 ul of recombinant lectins , Dectin-2-CRD-Alexa488 (provided by Dr Dimitra Lamprinaki) (4 µg/ml) in Flow Cytometry Staining Buffer (**FACS Buffer**) (Hanks' Balanced Salt Solution (HBSS) (Lonza, Switzerland) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1% BSA in the presence of 2 mM $CaCl_2$ or 10 mM ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetraacetic acid (EGTA) or with Gal-3 (10 µg/ml; **see section 2.2.1**) in PBS. Following centrifugation at 16,000 x *g* at 4°C for 5 min, the cells were harvested and washed with their respective incubation buffers. For Gal-3 binding, cells were incubated with mouse anti-His-tag antibody (2 µg/ml) (MilliporeSigma, USA) for 1 h at 4°C, centrifuged at 16,000 x *g* at 4°C for 5 min, then washed and finally incubated with the secondary antibody goat anti-mouse-IgG-APC (allophycocyanin) antibody (0.3 µg/ml) (BioLegend, UK) for 1 h at 4°C. The samples were then analysed by flow cytometry using a Fortessa flow cytometer (BD Biosciences, USA). The setting parameters were collected on a log scale and the

threshold of forward scatter (FSC) parameter was as set to 1000. The data were analysed using the FlowJo software version 5.7.1 (Tree Star, USA). The mean fluorescence intensity (MFI) is referred to the geometric mean fluorescence intensity value. The relative MFI (R-MFI) is referred to the value obtained after subtraction of the control MFI.

3.1.8.4 Cell Reporter assays

To assay the interaction between *L. reuteri* strains and BWZ.36 cells expressing murine CTLs, mDectin -1, mDectin-2, and SIGN-R1 reporter cells (Wittmann et al., 2016; Bene et. al., 2017) were grown in Roswell Park Memorial Institute Medium (RPMI) 1640 (Invitrogen, USA) containing 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, USA), 2 mM glutamine (Wako, Japan), 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. To assess the binding of *L. reuteri* strains to CTLs, 8 x 10^8 bacteria, grown in LDM II o OD_{600nm} of 1 were adsorbed onto 96-well microplates overnight at 4°C. Scleroglucan (10 µg/ml) (ELICITYL, France), furfurman (10 µg/ml) (ELICITYL, France), and lipopolysaccharides (LPS) from *Hafnia alvei* (10 µg/ml) (ELICITYL, France) were included as controls as specific ligands for mDectin1, mDectin-2 and SIGN-R1, respectively. Wells were washed with PBS, and BWZ.36 cells (expressing C-type lectins or mock cells) (5 × 10^5 cells/ml) were added to each well and incubated for 18 h at 37°C in a 5% CO_2 incubator. The microwell plates were then centrifuged at 510 × *g* for 3 min and the supernatant was discarded. The β-galactosidase activity was determined following the addition of 100 µl of 150 mM chlorophenol red-β-D-galactopyrasonide (CPRG; Roche) diluted in a chlorophenol red-β-D-galactopyranoside (CPRG) assay reaction buffer (PBS supplemented with 0.125% Triton X-100 and 100 mM 2-mercaptoethanol, Lonza, Switzerland) to each well. The plate was incubated for 45 min in a 37°C, 5% CO2 incubator prior to measurement of colour development (A570/630 nm) using a Bio-Rad Benchmark Plus microtiter plate reader.

3.1.8.4 Polarised *In Vitro* Organ Culture (pIVOC)

To assay the binding of *L. reuteri* strains to murine forestomach, the pIVOC model was established using methods based on Tsilingiri *et al* 2013. Briefly, forestomachs were harvested from specific pathogen-free (SPF) mice, cut open and washed three times with Ca^{2+} and Mg^{2+} free sterile PBS. A cloning cylinder (Bellco Glass, USA) was glued to the forestomach tissue using Vetbond Tissue Adhesive (3M). The centre well of an organ culture dish (Corning, USA), was overlaid with a metal grid, 1 ml Dulbecco's Modified Eagle Medium (DMEM) added to the centre well, and the forestomach placed onto the metal grid and 8 x 10^8 bacteria, grown in LDM II o OD_{600nm} of 1 were added to the cylinder in 25 µl DMEM for 1 h in an incubator at 5% CO_2 atmosphere at 37°C **(see Figure 27A)**. The forestomach biopsies were then removed

from the Snapwell support, washed three times in PBS to remove non-adherent bacteria. Adherent bacteria were quantified by lysing cell monolayers in 1% Triton X-100 for 15 min and plating out serial dilutions of lysates onto MRS agar plates. MRS plates were incubated at 37°C and colony forming units (CFU) were determined next day.

3.2 Protein purification and analysis

3.2.1 Expression and purification of recombinant Gal-3

E. coli BL21(DE3) cells expressing Galectin-3-his-tag (in-house, Leclaire et.al., 2018) were cultured in 1 l of LB containing 50 μg/ml carbenicillin. Cells were harvested by centrifugation at 6,000 × *g* at 4°C for 15 min and following a wash with PBS the pellets kept at -80°C until use. The cell pellets were suspended in ice-cold PBS (35 ml) and sonicated 10 times for 15 s each with 30 s incubation on ice in between each sonication step. After sonication, the solution was centrifuged at 8,000 x *g* at 4°C for 15 min, and the process was repeated 3 times. After each centrifugation, the supernatant was collected and passed through a 0.2 μM filter. Gal-3 was first purified by immobilised metal affinity chromatography (IMAC) using a Nickel-immobilised column (Bio-Rad, USA) using the buffers listed in **Table 4**. The eluate (10 ml) was then subjected to a second affinity chromatography using a lactose-agarose column (in house) using PBS and lactose buffer (150 mM lactose in PBS) as a washing buffer and elution buffer, respectively, and fractions (1 ml) were collected and stored at 4°C until use.

Table 5: IMAC buffer compositions.

IMAC SOLUTION	Tris-HCl	NaCl	Imidazole
Binding Buffer	20mM	500mM	5mM
Wash Buffer 1			20mM
Wash Buffer 2			40mM
Wash buffer 3			60mM
Elution Buffer			1M

3.2.2 MUB and tMUB purification

Native MUB or truncated MUB (tMUB) were purified from *L. reuteri* ATCC 53608 and *L. reuteri* 1063N, respectively. The bacteria were inoculated from −80°C glycerol stocks into MRS under anaerobic and static conditions for 16 h at 37°C, followed by sub-culture at 0.1% (v/v) for 24 h at 37°C to stationary phase. Cells were pelleted by centrifugation at 10,500 × *g* for 15 min at 4°C. MUB/tMUB proteins from the supernatant were precipitated using 60% ammonium sulphate overnight at 4°C. Following centrifugation at 10,500 × *g* for 15 min at 4°C, the pellet was resuspended in ddH_2O, and proteins were

extracted in a three-phase partitioning system using tert-butanol and an increasing concentration of ammonium sulphate. Ammonium sulphate was again added to the sample to a final concentration of 20% and one vol of tert-butanol was added to the sample. After thorough mixing by vortexing, the sample was centrifuged at 5000 x *g* for 10 min. The upper phase was removed, the protein pellet in the interphase was recovered and dissolved in ddH_2O, and CHAPS (3-[(3cholamidopropyl) dimethylammonio]1-propanesulfonate) was added at 0.5% w/v final concentration. MUB and tMUB were further purified by gel permeation chromatography (GPC), using a Superose 6 10300 GPC column (GE Healthcare, UK). PBS supplemented with 0.5% CHAPS was used as the elution buffer, at a flow rate of 0.4 ml/min. Fractions (1 ml) were collected and analysed by SDS-PAGE (**see section 2.2.5**). Before use, fractions containing MUB or tMUB were pooled and buffer-exchanged into PBS using Vivaspin 6 spin filters (100 kDa; Sartorius Stedim Ltd, UK) to remove any trace of CHAPS.

3.2.3 SRRP purification

Native SRRP, $SRRP_{100\text{-}23}$ and $SRRP_{53608}$ were purified from of *L. reuteri* 100-23 and *L. reuteri* 53608, respectively. An overnight culture of *L. reuteri* 100-23 or *L. reuteri* 53608 grown in MRS was used to inoculate 1 l of LDM-II, supplemented with 2% maltose. The culture was incubated overnight at 37˚C and the cells were removed by centrifugation at 10,000 x *g* for 10 min. The supernatant was then dialysed using a 100 kDa MWCO dialysis membrane (Thermofisher Scientific, UK) against 50 mM ammonium bicarbonate for 48 h, with 4 changes of buffer. The dialysed sample (~10 ml) was loaded onto an agarose-bound wheat germ agglutinin (WGA) affinity column (Vector labs, UK), pre-conditioned with 10 vol of washing buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 150 mM NaCl). Following loading with sample containing SRRP, the column was then washed with 5 vol of washing buffer. Bound $SRRP_{53608}$ or $SRRP_{100\text{-}23}$ were eluted with 3 vol of the washing buffer supplemented with 0.5 M *N*-acetyl-glucosamine (GlcNAc). The eluent fractions were dialysed using a 100 kDa MWCO dialysis membrane against 50 mM ammonium bicarbonate for 48 h at 4°C to remove the excess of GlcNAc and then stored at -20°C.

3.2.4 Protein quantification

Fractions from each protein purification were concentrated using a Vivaspin 2 or Millipore concentrator of 10 KDa MWCO. Proteins were quantified by Nanodrop (Thermo Fisher Scientific, UK).

3.2.5 SDS-PAGE

Protein samples were prepared by adding lithium dodecyl sulphate (LDS) and NuPAGE reducing agent, according to manufacturer's instructions (Invitrogen, USA). The samples were boiled at 70°C for 10 min

and then loaded onto a 4-12% Bis-Tris NuPAGE polyacrylamide gel (ThermoFischer Scientific, UK). HiMarkTM Unstained High Molecular Weight Protein Standard (Cell Signalling Technology, UK) was used as a protein marker. Following electrophoresis in 3-(N-morpholino) propanesulphonic acid (MOPS) SDS running buffer (50 min at 200 V constant voltage), the gels were stained with the Colloidal Blue staining kit (Life Technologies Ltd., UK).

3.2.6 Western Blotting

Proteins from SDS-NuPAGE gels were blotted onto an Amersham™ Hybond® P Western blotting Polyvinylidene difluoride (PVDF) membrane (GE Healthcare Lifesciences, UK), using the XCell II Blot module (ThermoFischer Scientific, UK), following the manufacturer's instructions with the following modifications. No methanol was used in the transfer buffer and transferring was performed for 2 h for large proteins (> 300 kDa). The membrane was blocked with Pierce protein-free PBS blocking buffer (ThermoFisher Scientific, UK) for 1 h and then immersed into 10 ml PBS blocking buffer supplemented with the primary antibody at appropriate concentration for 1 h (**Table 3**). The probed membrane was then washed three times with PBS, supplemented with 0.1% Tween®20 (PBST) and then probed with the appropriate secondary antibody (**Table 5**), before being washed again three times with PBST. When horseradish peroxidase (HRP)-conjugated antibody was used, the blots were visualised by the addition of the chromogenic 1-step 3,3',5,5'- Tetramethylbenzidine (TMB) blotting substrate solution (ThermoFisher Scientific, UK), until bands appeared. The blot was then washed extensively with ddH_2O. When alkaline phosphatase (AP)-conjugated antibody was used, the membrane was first washed once with 10 ml Tris-HCl 0.1 M, pH 9.6, before 10 ml of the visualisation solution (40 µM MgCl2, 0.1 mM nitroblue tetrazolium, 0.1 mM 5-bromo-4-chloro-3indolyl-phosphate toluidine) was added until bands appeared. The blots were then washed extensively with ddH_2O. After colour development, the blots were air-dried and then scanned on a GS-800 calibrated densitometer (Bio-Rad, UK). Alternatively, PBS blocking buffer supplemented with 5 µg/ml of an appropriate fluorescein isothiocyanate FITC- labelled lectin (**Table 6**) was used instead of primary antibodies for 1 h. The membrane was then washed with PBST three times and scanned using a Pharos-FXTM Plus molecular imager (Bio-Rad, UK), using excitation and emission wavelengths of 495 and 520 nm, respectively.

Table 6: Antibodies used in this study.

Antibody	Target	Supplier	Dilution
Rabbit anti-MUB_{53608}-R5 polyclonal ab	MUB53608-R5 repeat	BioGenes, Germany	1:100
Rabbit anti-$SRRP_{53608}$ polyclonal ab	Binding region of $SRRP_{53608}$	BioGenes, Germany	1:100
StrepMAB-Classic-HRP conjugate	Strep-tag II peptide	BA lifesciences, Germany	1:250
Goat anti-rabbit antibody, AP conjugate	Rabbit antibodies	Sigma Aldrich, UK	1:1000
Anti-mouse antibody, HRP conjugate	Mouse antibodies	Jackson laboratories, USA	1:1000

Table 7: Fluorescein-labelled plant lectins used in this study.

Lectin	Target	Supplier	Dilution
Wheat germ agglutinin (WGA)	GlcNAc or sialic acid	BioGenes, Germany	1:500
Concanavalin A (ConA)	α-mannose	BioGenes, Germany	1:500
Ricinus communis agglutinn (RCA)	Gal or GalNAc	ThermoFisher Scientific, UK	1:500
Griffonia simplicifolia Lectin I isolectin B4 (GSL-I B4)	α-Gal	BioGenes, Germany	1:500

3.2.7 Slot blot

Slot blot assays were used to assay the binding between purified human intelectin-1 (hINTL-1 provided by Professor Laura Kiessling, Massachusetts Institute of Technology, USA) and tMUB purified as described in **section 2.2.2**). Briefly, hINTL-1 (50 ug/ml) and BSA (used as a control) (1mg/ml) in PBS buffer (pH 7.4) were vacuum-blotted onto an Immobilon-P polyvinylidene difluoride membrane (3.8 × 11.6 cm, 0.45 µm, Millipore) using a Hoefer PR600 24-slot apparatus. 1–20 µg of tMub was loaded per slot in a total volume of 100 µl. Blots were blocked for 18 h by gentle rocking at RT in 10 ml of Thermoblock protein-free blocking agent in PBS buffer (ThermoFisher Scientific, UK). All subsequent washing steps were carried out with 20 ml of PBS buffer containing 0.05% (v/v) Tween 20. Blocked membranes were incubated at RT with 10 ml of fluorescein-conjugated tMUB protein (200 µg/ml f-

MUB, fluorescein/protein (F/P) ratio: 0.99–2.37) with gentle rocking in the dark for 20 h. Following excitation at 488 nm, fluorescein signals were detected at 530 nm in a Pharos FX™ Plus Molecular Imager (Bio-Rad) and quantified using Quantity One® v4.6.1 software (Bio-Rad)

3.2.8 Atomic force microscopy (AFM)

The interactions between MUB_{53608} and h-INTL1 was examined by covalently attaching lectin molecules to AFM tips and MUB_{53608} to the glass slides to enable binding interactions to be measured in a specific manner. Silicon nitride AFM tips (PNP-TR, Switzerland) and freshly cleaned glass slides were functionalized using a four-step procedure: the first step involved incubation in a 2% solution of 3-mercaptopropyltrimethoxy silane (MTS, Sigma– Merck, UK) in toluene (dried over a 4Å molecular sieve) for 2 h, followed by washing with toluene and then chloroform. In the second step, the silanised tips were incubated for 1 h in a 1 mg/ml solution of a heterobifunctional linker: MAL-PEG-SCM, 2 kD (Creative PEGWorks, USA) in chloroform. The silanised glass slides were incubated in 5 mM N g -maleimidobutyryl-oxysuccinimide ester (GMBS) in ethanol (ThermoFisher Scientific, USA). The tips and slides were rinsed with chloroform/ethanol, respectively, and then dried with argon. The third step involved covalent attachment of the lectins by incubation of the tips/slides in 1 mg/ml solutions of the proteins in PBS at pH 7.4 for 1 h at 21^{o}C, followed by a PBS washing step. The fourth step involved incubation of the functionalised cantilevers/slides in a 10 mg/ml solution of glycine in PBS to 'amine'-cap any unreacted succinimide groups, followed by washing in PBS. Binding measurements were carried out in PBS using a MFP3D BIO AFM (Asylum Research Inc., USA). The experimental data were captured in 'force-volume' (FV) mode at a rate of 2 mm/s in the Z direction and at a scan rate of 1 Hz and a maximum load force of 150-300 pN (pixel density of 32x32). Adhesion in the force spectra were quantified using a bespoke Excel macro which fits a straight line to the baseline of the retract portion of the force-distance data.

3.2.9 Proteomics analysis

3.2.9.1 Preparation of cell wall extracts, soluble cytoplasmic extracts and spent medium extracts

L. reuteri strains were grown to early stationary phase in LDMII media, and cells were harvested by centrifugation at 5,000 x *g* for 10 min. The spent growth medium was retained and concentrated using a Vivaspin-2 ultrafiltration spin column with a 100,000 Da molecular weight cut (Sartorius Stedim Biotech, France) at 4°C, following the manufacturer's instructions. The concentrated spent medium was then

buffer-exchanged with PBS and centrifuged to approximately 50-70-fold concentration. Soluble cytoplasmic extracts were prepared from PBS-washed bacteria as previously described in (Kankainen et. al., 2009) with the following modifications. Briefly, bacterial pellets from each culture were disrupted by vortexing with 100-200 µm diameter glass beads (Sigma- Merck, UK) for 50 s pulses at full speed with 2 min intervals on ice between pulses. The supernatant was then recovered and centrifuged at 16,000 x *g* for 30 min at 4 °C to pellet the cell wall material while the supernatant was kept as the soluble cytoplasmic extract.

3.2.9.2 Cell surface proteome extraction

Bacteria grown to early stationary phase in LDMII were harvested at 12 000 x *g* for 15 min at 4°C and washed with 'shaving' buffer (20 mM Tris-HCl, 150 mM NaCl pH 7.4). Cell pellets were resuspended in 0.5 ml of trypsin solution (10 µg/ml trypsin, 1 M D-arabinose, 10 mM CaCl2, 20 mM Tris-HCl, 150 mM NaCl pH 7.4) and incubated at 37 °C for 30 min with gentle shaking (10 rpm). Cells were then centrifuged at 17,000 x *g* for 20 min at RT, and the supernatant was collected, filtered (0.2 µm), snap frozen on dry ice and stored at 20°C. Shaved protein extracts were further digested with trypsin as follows. Samples were diluted in digestion buffer 1 (DB1: 8 M Urea 100 mM Tris, pH 8, 5 mM DTT) to a volume of 50 µl and incubated at RT for 2 h before 389 µg iodoacetamide (IAA) was added to each sample, followed by a 20 min incubation in the dark at RT. Ammonium bicarbonate (50 mM) was added to reduce the urea concentration to 1 M, and trypsin or chymotrypsin was added to the samples to obtain an enzyme: protein ratio of 1:50. Samples were incubated at 28°C, 16 h and then adjusted to 0.1 % (w/v) with trifluoracetic acid using a 2.5 % stock solution. The solution was filtered by OMIX Varian c18 tips (Walnut Creek, USA) following the manufacturer's instructions. The samples were dried using a speed vac (ThermoFisher Scientific, USA) and frozen at -80°C.

3.2.9.3 Mass Spectrometry Protein Identification

Protein bands of interest were excised from SDS-NuPAGE gels and cut up into small cubic pieces. The gel plugs were washed twice with 200 µl of ABC buffer (200 mM aqueous ammonium bicarbonate in 50% acetonitrile: ACN) for 15 min and then twice with ACN for 10 min at RT. The excess of ACN was removed and the gel plugs were air-dried for 15 min. A DTT solution (200 µl, 10 mM in 50 mM ammonium bicarbonate) was added and the samples were incubated at 60°C for 30 min. Then, the DTT solution was removed by dialysis and, upon addition of 200 µl iodoacetamide (IAA) solution (10 mM in 50 mM ammonium bicarbonate), the plugs were incubated at RT in the dark for 30 min. The IAA solution was removed by dialysis and the washing steps were repeated. Trypsin Gold (10 µl; 10 ng/µl; Promega, UK) was

added to the gel plugs along with an equal amount of 10 mM ammonium bicarbonate. After incubation at 37°C for 3 h, 20 µl of 1% formic acid was added, and the samples were further incubated in this solution at RT for 10 min. The suspension was then transferred to a clean tube and tryptic peptides were further extracted from the gel plugs by addition of 40 µl of 50% ACN and incubation for 10 min at RT. The samples were then dried on a Vacufuge® Plus vacuum concentrator (Eppendorf, UK). The peptide mixtures were analysed by LC MS/MS, using anOrbitrap Fusion trihybrid mass spectrometer coupled with a nano flow UHPLC system (ThermoFischer Scientific, UK) at the John Innes Centre (Norwich) by Dr Gerhard Saalbach. The peptides were separated on a C18 pre-column, using a gradient of 3-40% ACN in 0.1% formic acid (vol/vol), over 50 min at a flow rate of 300 nl/min, at 40°C. The peptides were fragmented in the linear ion trap by a data-dependent acquisition method, selecting the 40 most intense ions. Mascot (Matrix Science, UK) was used to analyse the raw data against an in-house maintained database of the *L. reuteri* proteomes. The tolerance on parent ions was 10 ppm and on fragments was 0.5 Da. Carboxymethylation of cysteine was selected as a fixed modification and oxidation of methionine as a variable modification. Samples were analysed by Dr Dimitris Latousakis using LTQ-Orbitrap, the resulting RAW files were converted to Mascot generic files using Proteome Discover version 1.1.0.263 (ThermoFisher Scientific, USA) prior to protein identification using the MS/MS ion search with a Mascot search engine (Matrix Science Ltd., UK).

3.4 Glycan analysis

Monosaccharide composition analysis by GC-MS chromatography

For the preparation of the samples, 100 µg of SRRP was precipitated by addition of 1.33 vol of chloroform and 2.67 vol of methanol and resuspended in ddH_2O. Myo-inositol (2.5 µg) used as an internal reference was added to the suspension, which was then lyophilised. The dried sample was resuspended in 0.5 ml 1N methanolic HCl and incubated at 80^{o}C for 16 h. The methanolysed sample was cooled down to 25^{o}C and silver carbonate (~50 mg) was added, followed by 100 µl addition of acetic anhydride. The N-acetylation reaction was incubated at RT for 24 h. The sample was centrifuged at 3,000 x *g* for 5 min and the supernatant was transferred into a clean vial and dried under a gentle stream of nitrogen. Tri-Sil HTP reagent (200 µl; ThermoFischer Scientific, UK) was added to the sample and the derivatisation reaction was incubated at 80°C for 30 min. The sample was dried again under nitrogen, resuspended in 1 ml hexane and sonicated for 15 min. After centrifugation at 3,000 x *g* for 5 min, the supernatant was transferred into a clean vial and dried under nitrogen. The dried sample was dissolved into 100 µl dichloromethane and transferred to a GC-compatible vial. The samples were then

analysed on an Agilent 7890B GC-MS system paired with an Agilent 5977A GC-MS detector (Agilent Technologies, UK), using an BPX70 column (SGE Analytical Science, Australia). Helium was used as the carrier gas. The inlet was maintained at 220^{o}C, 12.9 psi, and 23 ml/min flow. The injection volume was 1 µl in split mode (1:20). The oven temperature was increased from 100^{o}C to 120^{o}C over 5 min, followed by a second increase from 120^{o}C to 230^{o}C over 40 min. Data processing was performed using MassHunter Qualitative Analysis B.07.00 (Agilent Technologies, UK).

3.5 Immunological assays

3.5.1 Blood samples

Human peripheral blood was obtained from haemochromatosis patients undergoing a therapeutic venesection at the Norfolk and Norwich University Hospital (Norwich, UK). Blood collection in this study was approved by the Faculty of Medicine and Health Sciences Research Ethics Committee REC reference number 2013/2014 -14HT (University of East Anglia, UK).

3.5.2 Human monocyte-derived DC (moDC) Cultures

Human blood monocytes were isolated following centrifugation of whole blood samples (vol?) using a Ficoll-Paque gradient (GE Healthcare, UK) for 40 min, at 400 x *g* and RT with minimum acceleration and deceleration speed. The peripheral blood mononuclear cells (PBMCs), located at the interface between the serum and granulocytes were collected. PBMCs were diluted 3-fold with Hanks' Balanced Salt Solution (HBSS) (ThermoFisher Scientific, USA) supplemented with 3% faetal bovine serum (FBS) and 10 mM EDTA and the solution centrifuged at 270 x *g*, RT for 7 min. Monocytes ($CD14^{+}$ cells) were isolated from PBMCs by magnetic bead separation, using CD14 positive selection microbeads (StemCell technologies or ThermoFisher, USA) according to the manufacturer's instructions. MoDCs were generated by culturing 10^{6} cells/ml of fresh isolated monocytes in Mercedes medium (RPMI 1640 medium (Lonza, Switzerland) supplemented with 25 mM HEPES, 10% HI FBS (ThermoFisher Scientific, USA), 55 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza), 2 mM glutamine (Lonza, Switzerland), 1 mM non-essential amino acids (Lonza, Switzerland) and 1 mM Sodium Pyruvate (Lonza, Switzerland) supplemented with cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (PeproTech, UK) (25 ng/ml) for differentiation of monocytes to moDCs. The cells were incubated for 7 days at 37^{o}C in a 5% CO2 incubator with change of medium and cytokine supplementation on day 3.

3.5.3 Stimulation of human primary myeloid cells with bacteria

L. reuteri strains were grown in LDMII media for 18 h under reaching stationary conditions at 37°C. Bacterial suspensions were washed with sterile PBS buffer three times and OD_{600} nm was measured by spectrophotometry and converted to cell/ml. Human moDC cultures were stimulated using 1 µg/ml LPS (ultrapure LPS, InvivoGen, USA) in 100 ul HBSS solution as a control or with heat-inactivated *L. reuteri* strains in HBSS at a non-toxic ratio of 5:1 bacteria:moDCs and were co-cultured for another 16 h in an incubator at 37^{o}C atmosphere containing 5% CO_2. On the next day, cells were centrifuged at 500 x *g* for 3 min and the supernatant collected and stored at -80^{o}C for later use (cytokine analysis) while the cell pellet was used immediately for cell surface marker expression analysis.

3.5.4 Cytokine analysis

Cell culture supernatant was monitored for human IL-1β, IL-6, IL10, IL-13, IFNγ, TNFα and IL-8 production by either ELISA (BioLegend, UK) or ProcartaPlex-12 plex (ThermoFisher Scientific, USA) analysis according to the manufacturers' instructions. Multi-plex readings were performed on Luminex® 100/200™.

3.5.5 Isolation and culture of mononucleocytes from mouse bone marrow

C57BL/6 mice were maintained at the Disease Modelling Unit (DMU) of the University of East Anglia (Norwich, UK). C57BL/6 were killed by cervical dislocation and disinfected in 75% ethanol for 5 min. The tibias and femurs were removed under sterile conditions, then soaked into RPMI-1640 medium (Sigma-Merck, UK) supplemented with 1% FBS. Both ends of the bone were cut off with scissors, and the needle of a 1 ml syringe was inserted into the bone cavity to push the bone marrow out of the cavity into a sterile culture dish with RPMI-1640 medium. The bone marrow cells in the dish were collected and centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. The pellet was resuspended with red blood cell (RBC) lysis buffer (0.8% NH4Cl, 0.1 mM EDTA in water, buffered with KHCO3 to achieve a final pH of 7.2 - 7.6) (Lonza, Switzerland) to lyse the RBCs. Following a second centrifugation step under the same conditions, the supernatant was discarded, and the pelleted cells were washed with PBS and collected. Isolated monocytes were cultured in 100 mm × 20 mm cell culture dishes at a density of 5.0 × 10^5 cells/ml in Gibco's serum-free AIM-V medium (Invitrogen, USA) supplemented with 80 ng/ml granulocytemonocyte stimulating factor (GM-CSF) (Gentaur Molecular Products, Belgium) in an incubator at 37^{o}C atmosphere containing 5% CO_2.

3.5.6 Bone marrow-derived dendritic cells (BMDCs) stimulation

BMDCs cultured as described in **section xx**, were used for stimulation assays with bacteria. *L. reuteri* strains were grown in LDMII media for 18 h at 37°C under static conditions. Bacterial suspensions were washed with sterile PBS buffer three times and OD_{600} nm was measured by spectrophotometry. Mouse BMDC cultures were stimulated with LPS as a control (1 µg/ml ultrapure LPS in PBS, InvivoGen, USA) or with heat-inactivated *L. reuteri* strains at a non-toxic ratio of 100:1 bacteria:BMDCs and were co-cultured for another 16 h in an incubator at 37^{o}C atmosphere containing 5% CO_2.

3.5.7 Flow cytometry analysis

Phenotyping of resting and activated BMDCs was performed by flow cytometry using mouse anti-HLA-DR-PE (BD Biosciences, UK) (1:1000), anti-CD80-FITC (BD Biosciences, UK) (1:1000) and anti-CD83-FITC (BD Biosciences, UK) (1:1000) and isotype-matched control antibodies (R&D Systems, USA) (1:100). Briefly, $5x10^5$ BMDCs in Gibco's serum-free AIM-V medium (Invitrogen, USA) were incubated with *L. reuteri* strains at a non-toxic ratio of 100:1 bacteria:BMDCs and were co-cultured for another 16 h in an incubator at 37^{o}C atmosphere containing 5% CO_2. Fluorescence intensities were measured using FACS Calibur (BD Biosciences, USA). The data were analysed using the FlowJo software version 5.7.1 (Tree Star, USA).

3.5.8 Imaging Flow Cytometry analysis

Imaging flow cytometry analysis was performed using an Amnis Imagestream in standard configuration, equipped with 405 and 488 nm lasers for excitation and a 785 nm laser for a scatter signal with standard filter sets, multimagnification (20×/40×/60×) and extended depth of field. The INSPIRE software (Amnis, USA) was used for acquisition and IDEAS software for analysis. The machine was fully calibrated and passed all tests prior to each acquisition of samples using the machines calibration and test scripts. Imaging flow cytometry analysis was used to assess the internalisation of *L. reuteri* strains by BMDCs. BMDCs were differentiated in cell culture dishes at a density of 5.0×10^5 cells/ml in medium supplemented with GM-CSF for 7 days (**See section 2.5.6**). BMDCs were then stained with Cell Trace Violet (CTV) for 15 minutes at RT and stimulated with CFDA labelled *L. reuteri* strains. Co-culture of CTV stained BMDCs and CFDA labelled bacteria was then performed at 37^{o}C (unless stated otherwise) for 1 hour. Single stain compensation tubes for each stain was used and unstained tube was prepared alongside test samples. Cells were filtered through 35 µm nylon cell strainer mesh tubes (BD Biosciences, USA) directly prior to acquisition. A minimum of 10,000 events per sample were acquired. Compensation matrices were generated by running single stained particles or cells (i.e., single cell

surface marker) and analysed using IDEAS software. Briefly, cells were first plotted as area versus aspect ratio of the brightfield images and a single cell gate drawn, followed by a focused gate based on intensity of the respective fluorophore channels. Bivariate dot plots were then used to plot single focused cells as the bright detail intensity of brightfield versus the bright detail intensity of darkfield and a region drawn to identify the cells. The measurement of internalisation was based on the Imagestream X (ISX) internalisation feature, defined as the ratio of intensity measured inside a cell to the intensity of the entire cell. Briefly, a mask eroded by 4 pixels from the brightfield cell image default mask was created and the bright detail intensity of darkfield measured within the eroded mask to enable the internalisation score. Internalisation high gates were drawn based on background values from the negative samples.

3.5.9 NF-κB reporter cell assay

THP-1 blue NF-κB reporter cells (InvivoGen, USA) were used for monitoring nuclear factor Kappa B (NF-κB) signal transduction pathways. These cells are stably transfected and express a secreted embryonic alkaline phosphatase (SEAP) reporter gene driven by an IFN-β minimal promoter fused to five copies of the NF-κB transcription factor, which promotes cytokine production. The translocation of NF-κB from the cytosol to the nucleus induces the release of the SEAP phosphatase, whose activity can be monitored colorimetrically. THP1-Blue™ NF-kB cells (grown to 90% density) were washed once with PBS and resuspended in RPMI-1640 media (Sigma- Merck, UK) without antibiotics at a density of 100,000 cells/ml in T-75 flask (Thermo Fisher Scientific, USA). 8 x 10^8 *L. reuteri* bacteria, cultured in LDM II, at OD_{600nm} of 1 were added at a MOI of 10:1, 50:1 and 100:1. Heat-killed *Listeria monocytogenes* (HKLM) (InvivoGen, USA), was used as a positive control at a MOI of 50:1. Cells were then incubated at 37°C in a 5% CO2 atmosphere for 8 h. 20 µL of supernatant was then removed from the cell culture, transferred into 96-well microplates and incubated with the QUANTI-Blue™ detection media (InvivoGen, USA) according to the manufacturer's instructions for 1 h and the OD measured at 620 nm. The experiments were carried out using technical replicates in at least three independent experiments.

3.6 Statistical analyses

All data are shown as means ± standard errors of the mean. Statistical analysis was performed using GraphPad Prism software (version 7). Student's T-test was used to determine differences between two groups. One-way ANOVA with Tukey's multiple comparisons test was used to determine differences between multiple groups. All gene expression data were log10 transformed before statistical analysis. A

P value of <0.05 was considered significant with degrees of statistical significance presented as follows:

* = P<0.05, ** = P<0.01, *** = P<0.001.

CHAPTER 4: THE ROLE OF ADHESINS IN THE INTERACTION BETWEEN *L. REUTERI* STRAINS AND THE HOST.

4.1 Introduction

L. reuteri is a Gram-positive facultative anaerobe and represents a natural inhabitant of the GI tract of a wide variety of vertebrates including rodent, birds, livestock and humans[476]. *L. reuteri* provides an excellent paradigm to study mechanisms for host-microbial symbiosis in vertebrates. *L reuteri* is one of the few lactic acid bacteria that have adapted to survive in the stomach and can grow in the presence of gastric acids and bile salts[477]. *L. reuteri* strains express exopolysaccharides (EPS) involved in colonisation and survival in the gut[387]. The occurrence of *L. reuteri* strains in breastmilk and the forestomach and small intestine is in line with their ability to preferentially utilise simple sugars available from the diet, rather than complex polysaccharides reaching the large intestine undigested[478]. The ecological strategies of *L. reuteri* are fundamentally different in humans and animals[30]. In rodents, pigs, chickens and horses, *L. reuteri* form large populations in proximal regions of the GI tract, and they adhere directly to the stratified squamous epithelium present at these sites[394]. In mice and rats, adherence occurs in the forestomach[479], and this process appears to be important with regards to the ecological fitness of the bacteria[480]. Bacterial adhesion to host surfaces is recognised as one of the main contributors of host colonisation and previous research identified *L. reuteri* adhesins such as SRRP (serine rich repeat protein)[378], MUB (mucus binding protein)[426] or mucus-binding protein A (CmbA) mediating host specialisation[433]. Here we will investigate the adhesion properties of *L. reuteri* strains characteristic of different host clades[376] focusing on wild-type (WT) and mutant strains of *L. reuteri* 100-23 (rodent isolate), ATCC 53608 (pig isolate) and ATCC PTA 6475 (human isolate).

L. reuteri strain 100-23 is an autochthonous inhabitant of the rodent gut[481]. *In vivo* experiments conducted with ex-*Lactobacillus*-free mice have unravelled the molecular processes that allow this bacterial strain to colonise the stratified squamous epithelium of the mice forestomach[482, 483]. Genomic features associated with the host specificity of rodent strains such as *L. reuteri* 100-23 include the aSec secretion system[31] or large cell surface proteins such as SRRP or the large surface protein (LSP)[483]. The specialised aSec system is encoded by genes that are normally co-located within a gene cluster and is composed of the motor protein SecA2, the translocon channel SecY2, two GT-encoding genes (most commonly called GtfA and GtfB) and three to five accessory sec proteins (Asp1-5)[442]. Using *L. reuteri* 100-23 *aSec* mutants, *SecA2, Asp2, GtfB*, as well as insertion mutants targeting cell surface proteins SRRP and LSP in germ-free mouse colonisation assays, epithelial association of the bacteria to the

forestomach was shown to be fully abrogated, indicating that initial adhesion represented the most significant step in biofilm formation, likely conferring host specificity[378]. Exopolysaccharide (EPS) produced by *L. reuteri* 100-23 has also been shown to influence colonisation of the murine gut[387]. Mutation of the *L. reuteri* 100-23 fructosyl transferase (*ftf*) gene, resulting in loss of EPS production[387], led to an impaired colonisation of the *L. reuteri* 100-23 *ftf* mutant strain in competition with the WT strain in murine GI tract.

pan-genome analysis of lactobacilli species isolated from pigs has been used to show that the *L. reuteri* ATCC 53608 colonises the porcine small intestine mucosa[484]. Analysis of the completed genome of the ATCC 53608 strain revealed the presence of an accessory SecA2–SecY2 secretion system with an associated SRRP ($SRRP_{53608}$) that shared the same domain organization as $SRRP_{100\text{-}23}$[394]. In the mammalian small and large intestine, mucus is the first point of contact between the gut microbiota and the host. Previous studies showed that binding of *L. reuteri* to mucus was strain-specific[388] and mediated by mucus binding proteins such as MUB in *L. reuteri* ATCC 53608[388, 434]. *L. reuteri* 1063N is a natural mutant of ATCC 53608 which lacks the LPxTG cell-wall anchor motif and thus the ability to attach to the cell surface[388]. *L. reuteri* 1063N was shown to display low mucus-binding and aggregation capacities, suggesting that the cell-wall-anchored MUB to such phenotypes [388]. In addition, MUB purified from *L. reuteri* ATCC 53680 was shown to bind to mucin by atomic force spectroscopy[485].

L. reuteri PTA 6475 isolated from human breast milk (www.biogaia.com) was originally named as "MM4-1A" (Mother's Milk from mother number 4, first sample, clone A). *L. reuteri* PTA 6475 is believed to colonise human GI tract[386] and molecular and structural studies revealed that this strain expresses a mucus adhesin called mucus-binding protein A (CmbA). CmbA recognises Caco-2 cells and mucus as determined using direct interaction between ATCC PTA 6475 strain and Caco-2 cells and mucus *in vitro*[433]. In addition, *L. reuteri* 6475-KO mutant was shown to have reduced binding to HT-29 cells and the goblet cell-derived cell line LS174T secreting intestinal mucin MUC2 compared to the WT strain *in vitro*[432].

Here we investigated the role of adhesins in the interaction between *L. reuteri* 100-23, ATCC PTA 6475 and ATCC 53608 strains to mucin and the epithelium *in vitro*.

4.2 Results

4.2.1 The role of surface adhesins in the interaction between L. reuteri strains and mucin.

4.2.1.1 Binding of *L. reuteri* strains to mucin is strain specific.

Binding to mucin was first assayed with an ELISA-type assay using cFDA-labelled *L. reuteri* WT and mutant strains against immobilised purified porcine mucin (pPGM) or BSA (negative control). The values were expressed as percentage binding values normalised to the mean value for each strain, used as internal controls.

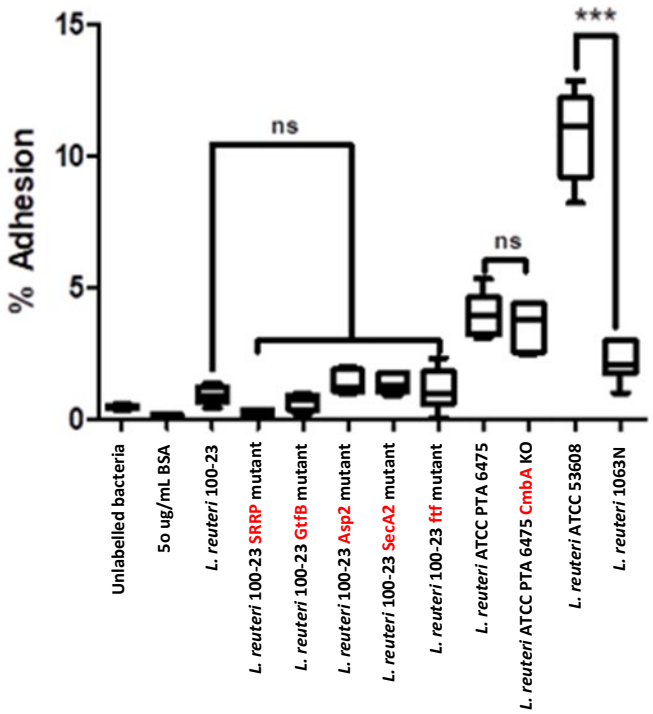

Figure 24: Binding of *L. reuteri* strains to mucin.

L. reuteri 100-23, ATCC PTA 6475 and ATCC 53608, as well as their mutants, were grown to early stationary phase, cF-labelled and added to pPGM or BSA coated plate. Following washing, bound bacteria were lysed with SDS in NaOH. The released cF- fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 485 and 520 nm as excitation and emission wavelengths, respectively. Each assay was performed in triplicate. Standards were included on each occasion also in triplicate consisting of SDS lysed cF-labelled bacteria to evaluate the % adhesion.

L. reuteri strains varied markedly in their ability to bind to mucin *in vitro*, with substantial differences reflected in percentages of adhesion, ranging from 0.045% (± 0.02 %) for *L. reuteri* 100-23 *SRRP* mutant to 12.8% (± 2.07 %) for *L. reuteri* ATCC 53608. In the strains isolated from rodents (**Figure 24**). There was no difference in binding to pPGM between the *L. reuteri* 100-23 WT strain and mutant strains lacking SRRP or proteins involved in its glycosylation transport or secretion as shown by the comparable levels of fluorescence detected between the strains (**Figure 24**). Furthermore, the *L. reuteri* 100-23 *ftf* mutant strain lacking EPS, showed similar levels of binding to pPGM compared to the WT strain (**Figure 24**).

The *L. reuteri* ATCC 53608 WT strain on the other hand, showed higher binding to pPGM compared to the MUB deficient 1063N mutant (**Figure 24**), in line with previous results showing that MUB is involved in the interaction to mucus[388, 426]. No binding was observed between any of the *L. reuteri* strains and BSA, confirming the specificity of the interaction to mucin.

4.2.1.2. Effect of flow conditions on *L. reuteri* ATCC 53608 strains binding to mucin

Here, to further investigate the role of MUB in the interaction of *L. reuteri* ATCC 53608 strain with mucin, a medium-throughput continuous flow system BioFlux 200 which mimics flow conditions of physiological liquids in the human body. The system was used to test binding of *L. reuteri* ATCC 53608 and 1063N cells in exponential phase to mucin coated onto the flow cell under shear flow conditions. Planktonic and weakly adhered cells were then flushed from the test section by applying a flow and the cells were inspected under an inverted microscope.

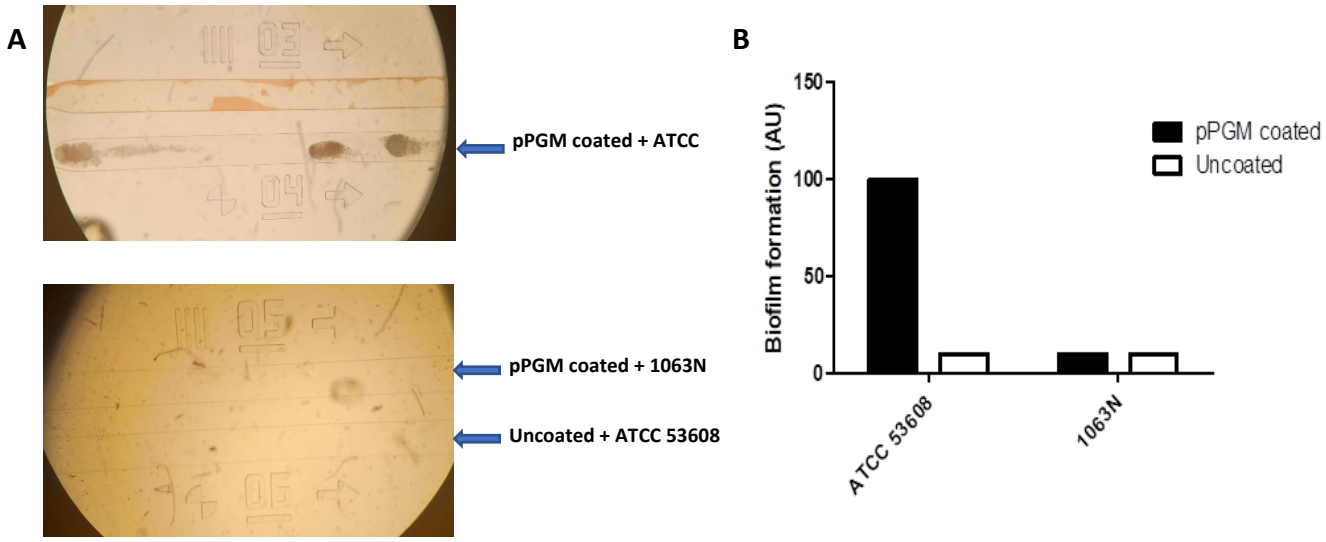

Figure 25: Binding of *L. reuteri* ATCC 53608 to mucin under flow.

(A) *L. reuteri* cultures at exponential phase were allowed to attach to the surface of glass coated or not with pPGM for 30 min in the absence of flow. Planktonic and weakly adhered cells were then flushed from the test section by applying a flow rate at 0.3 dyn (~40 µl/h) and the cells were inspected under an inverted microscope for the formation of colonies at 24 h. All microfluidic experiments were conducted at 37°C. (B) Image J was used to quantify relative biofilm formation between the two strains.

Large colonies of ATCC 53608 were apparent in the pPGM-coated cell whereas, no colonies or cells were observed in the un-coated cell used as a control (**Figure 25**). In contrast, the 1063N strain (which does not express MUB on its cell surface) could not be detected in the pPGM coated which confirms that MUB is important for *L. reuteri* ATCC 53608 to bind to mucin under both static and fluidic conditions.

4.2.1.3. Effect of GI stress conditions on *L. reuteri* ATCC 53608 strains binding to mucin

To gain a better understanding of *L. reuteri* ATCC 53608-mucin interactions in the gut, we next evaluated the adhesion ability of *L. reuteri* ATCC 53608 strain to mucin using conditions that recapitulate the simulated gastric and intestinal fluids. The bacterial cell suspensions were incubated in conditions that simulate the major factors influencing the survival of the ingested microorganisms across the GI tract. We have considered two relevant factors, the influence of acid pH values and the further action of bile salts and pancreatin which will be tested using simulated gastric juice (SGJ) and simulated intestinal fluid (SIF), respectively, simulating cumulative gastric delivery of bacteria to the intestine during GI transit.

Bacterial viability was assayed by plating samples collected after incubation in simulated gastric fluid and after incubation in simulated intestinal fluid. The percentage of bacterial survival was calculated as follows: (CFU_{assay} / $CFU_{control}$) x 100 where CFU_{assay} represents CFU after incubation in simulated gastric or intestinal fluids and $CFU_{control}$ the CFU after incubation in PBS as a control.

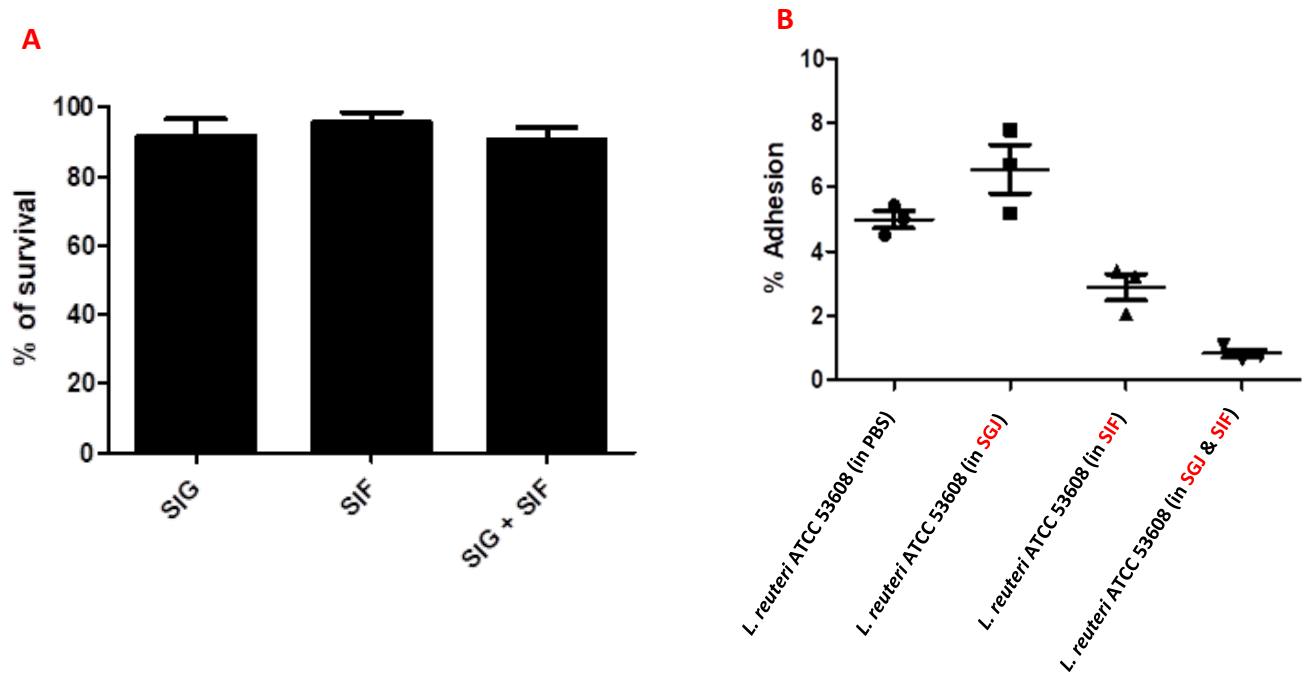

Figure 26: Effect of gastrointestinal conditions on binding of *L. reuteri* ATCC 53608 strain to mucin.

(A) Survival rates of *L. reuteri* ATCC 53608 strain after 1 h of exposure to simulated gastric and after a consecutive 2 h of exposure to simulated intestinal fluid. **(B)** *L. reuteri* ATCC 53608 bacteria were grown to early stationary phase, cF-labelled and added to pPGM or BSA coated plate. Following washing, bound bacteria were lysed with SDS in NaOH. The released cF -fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 485 and 520 nm as excitation and emission wavelengths, respectively. Each assay was performed in triplicate. Standards were included on each occasion also in triplicate consisting of SDS lysed cF-labelled bacteria to evaluate the % adhesion.

L. reuteri ATCC 53608 showed high survival rates in both simulated gastric and intestinal conditions as indicated by the comparable levels of CFU formation following simulated gut conditions (**Figure 26A**). Moreover, compared to the untreated sample*, L. reuteri* ATCC 536608 treated with SGJ showed, although not significant, slightly higher binding to pPGM (**Figure 26B**) whereas, bacteria treated with SIF and SGJ, or SIF showed reduced binding to pPGM (**Figure 26B**). These results suggest that gastric, but not intestinal stress, may cause changes in the bacterial surface leading to an increased ability to adhere to mucin.

To assess the effect of simulated gastric conditions on the expression of the cell surface proteins of *L. reuteri* ATCC 53608 strain, the surface expression of MUB_{53608} and $SRRP_{53608}$ was analysed by flow cytometry following immunodetection with polyclonal antibodies raised against these adhesins.

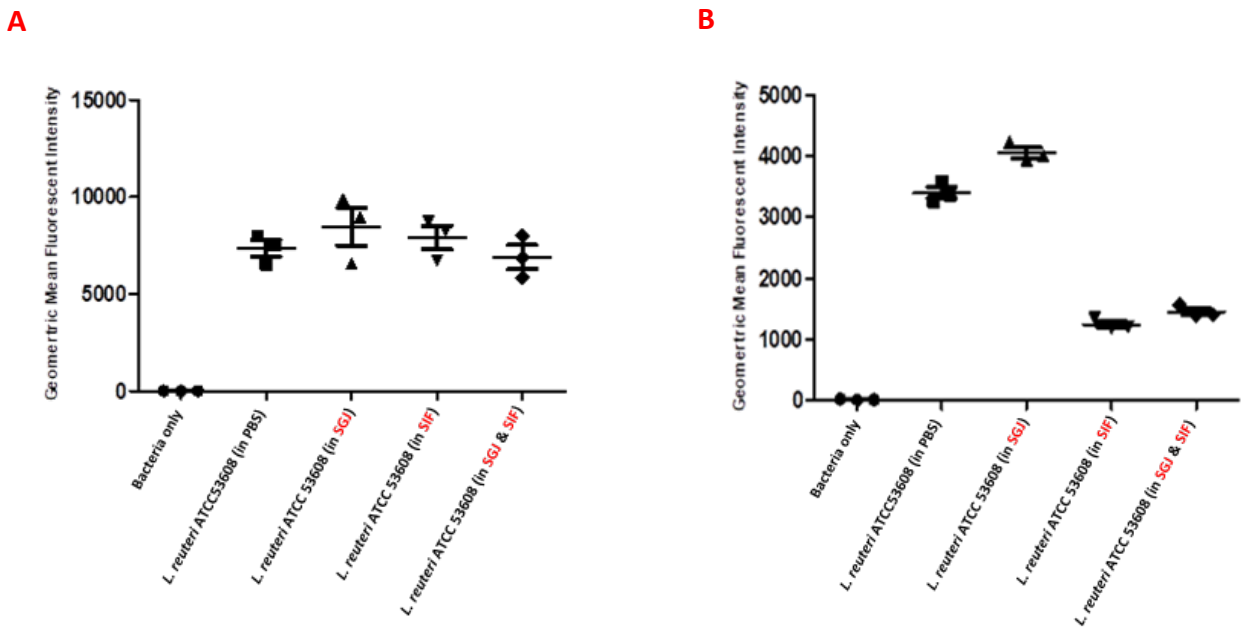

Figure 27: Effect of gastric conditions on the cell surface expression of MUB_{53608} and $SRRP_{53608}$.

L. reuteri ATCC 53608 bacteria were grown to early stationary phase and the surface expression of **(A)** MUB_{53608} and **(B)** $SRRP_{53608}$ was detected using primary anti-MUB (1:1000) or anti-SRR (1:500) and anti-rabbit secondary antibody (1:1000). Fluorescence intensities were measured by FACS Calibur (BDBiosciences, USA). The data were analysed using the FlowJo software version 5.7.1 (Tree Star, Ashland, USA).

Surface expression of MUB_{53608} was not affected following treatment of the ATCC 53608 strain with the simulated gastric conditions (**Figure 27A**). An increased expression of $SRRP_{53608}$ was detected following SGJ treatment (**Figure 27B**), whereas treatment of the bacteria with SIF or with a combination of SGJ and SIF reduced the surface expression of $SRRP_{53608}$ (**Figure 27B**), consistent with the reduction in mucin binding observed following treatment of the *L. reuteri* ATCC 53608 bacteria with simulated gastric condition **(Figure 26B).**

4.2.2 The role of surface adhesins in the interaction between L. reuteri and the epithelium

We next investigated the role of adhesins in the ability of *L. reuteri* 100-23, ATCC 53608 and ATCC PTA 6475 strains to adhere to the epithelium using the human colorectal adenocarcinoma cell lines (HT-29 MTX and HT-29 cells) and murine forestomach explants. HT29 cells are a pluripotent intestinal epithelial cells (IECs) and HT-29 MTX cells derived from HT-29 following treatment with methotrexate[486] produce MUC1, MUC3, MUC4 and MUC5C mucins which are predominantly expressed in the small and large intestine resulting in a patchy mucus layer[487, 488].

We first investigated the role of MUB in the interaction between the *L. reuteri* ATCC 53608 and IECs by comparing bacterial adherence of ATCC 53608 WT strain and MUB deficient strain 1063N to both HT-29 and HT-29 MTX cells.

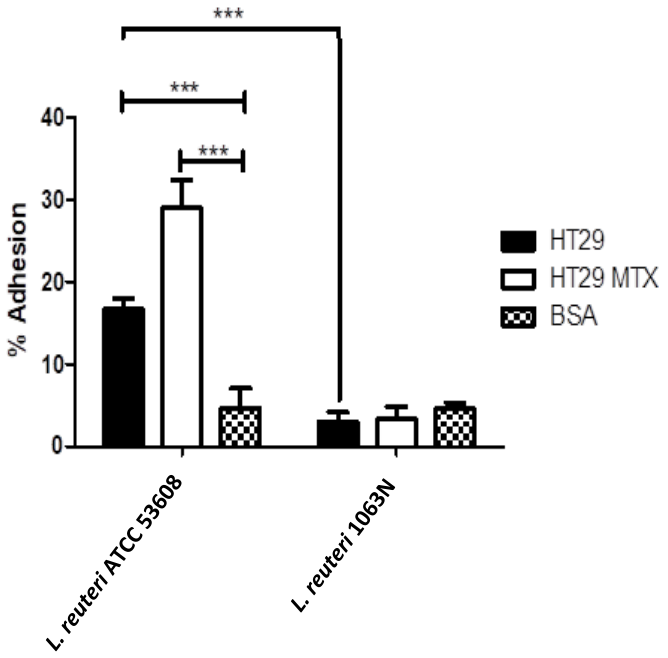

Figure 28: Adhesion of *L. reuteri* ATCC 53608 and mutant strain to IECs.

L. reuteri ATCC 53608 wild-type and mutant strain were grown to early stationary phase, cF-labelled and added to confluent cell layers of ~5×10^6 HT-29, HT-29 mtx or 1% BSA coated plate. Following washing, bound bacteria were lysed with SDS in NaOH. The released cF -fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 485 and 520 nm as excitation and emission wavelengths, respectively. Each assay was performed in triplicate. Standards were included on each occasion also in triplicate consisting of SDS lysed cF-labelled bacteria to evaluate the % adhesion.

We showed impaired binding of the MUB deficient 1063N strain compared to the ATCC 53608 WT strain to both the HT-29 and HT-29 MTX cells **(Figure 28).** In addition, adhesion of the ATCC 53608 strain was significantly higher to the mucus-producing HT-29 MTX cells compared to HT-29 cells, further supporting the role of MUB in the interaction between the bacteria and mucus.

We next determined the binding capacity of *L. reuteri* ATCC PTA 6475 WT and CmbA KO mutant strain to bind to IECs.

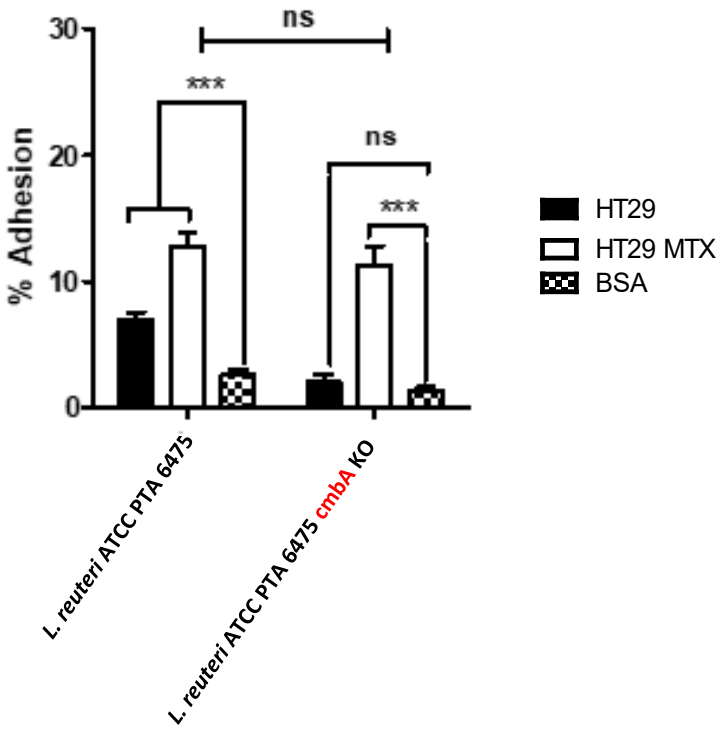

Figure 29: Adhesion of *L. reuteri* PTA 6475 and mutant strains to IECs.

L. reuteri ATCC PTA 6475 wild-type and mutant strains were grown to early stationary phase, cF-labelled and added to confluent cell layers of ~5×10^6 HT-29, HT-29 mtx or 1% BSA coated plate. Following washing, bound bacteria were lysed with SDS in NaOH. The released cF -fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 485 and 520 nm as excitation and emission wavelengths, respectively. Each assay was performed in triplicate. Standards were included on each occasion also in triplicate consisting of SDS lysed cF-labelled bacteria to evaluate the % adhesion.

The PTA 6475 WT strain showed higher binding to the HT-29 cells compared to the CmbA KO mutant strain (**Figure 29**), suggesting that CmbA contributes to the interaction between the bacteria and epithelial cells. The PTA 6475 WT and CmbA KO mutant also showed a comparable level of binding to the HT29 MTX, which may indicate that CmbA may not be involved in the interaction with mucus under the conditions tested.

Next, we investigated the ability of *L. reuteri* 100-23 WT and mutant strains to bind to HT-29 and HT-29 MTX cells.

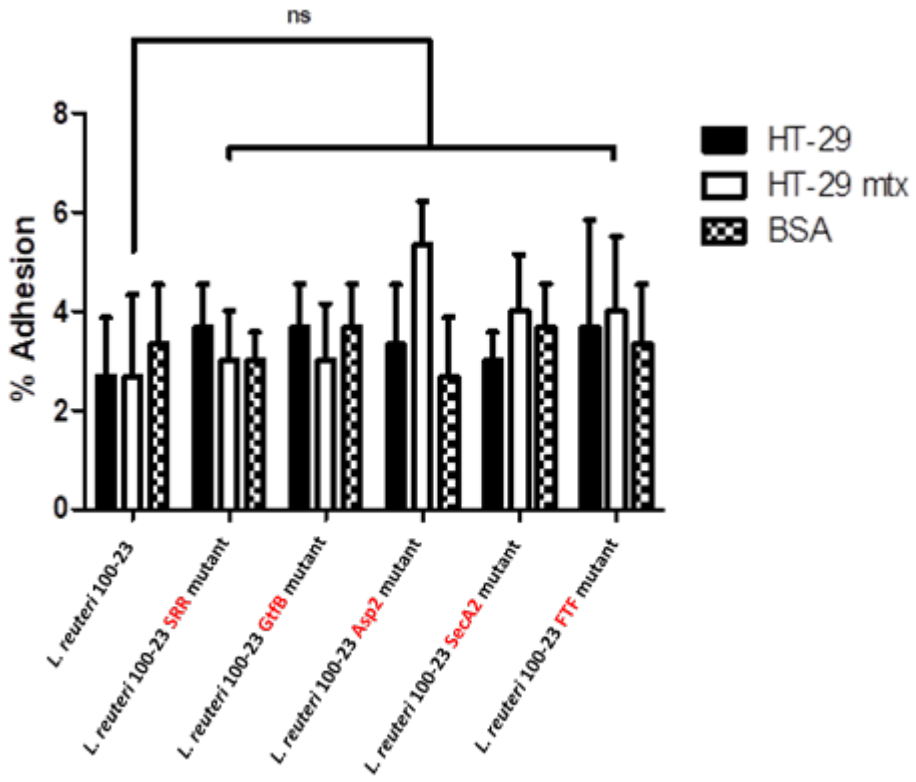

Figure 30: Adhesion of *L. reuteri* 100-23 and mutant strains to IECs.

L. reuteri 100-23 WT and mutant strains were grown to early stationary phase, cF-labelled and added to confluent cell layers of ~5×10^6 HT-29, HT-29 MTX or 1% BSA coated plate. Following washing, bound bacteria were lysed with SDS in NaOH. The released cF -fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 485 and 520 nm as excitation and emission wavelengths, respectively. Each assay was performed in triplicate. Standards were included on each occasion also in triplicate consisting of SDS lysed cF-labelled bacteria to evaluate the % adhesion.

The rat isolate *L. reuteri* 100-23 WT and *aSec* mutant strains did not bind to HT29 or HT29 MTX cells as shown by the comparable levels of binding observed between to the IECs and the negative control, BSA **(Figure 30)**. The *L. reuteri* 100-23 *ftf* mutant also revealed a comparable level of adhesion to HT-29 and HT-29 MTX cells compared with WT strain **(Figure 30)**.

Since the *L. reuteri* 100-23 strains did not bind to the human colon cancer cell lines, we next investigated the ability of these strains to bind to the forestomach epithelium using a polarised *in vitro* organ culture (pIVOC) based on forestomach explants from specific-pathogen-free (SPF) mice (**Figure 31**). The use of a polarised system enables to restrict bacterial access to a defined surface area and therefore allows quantification of bacterial adhesion[489].

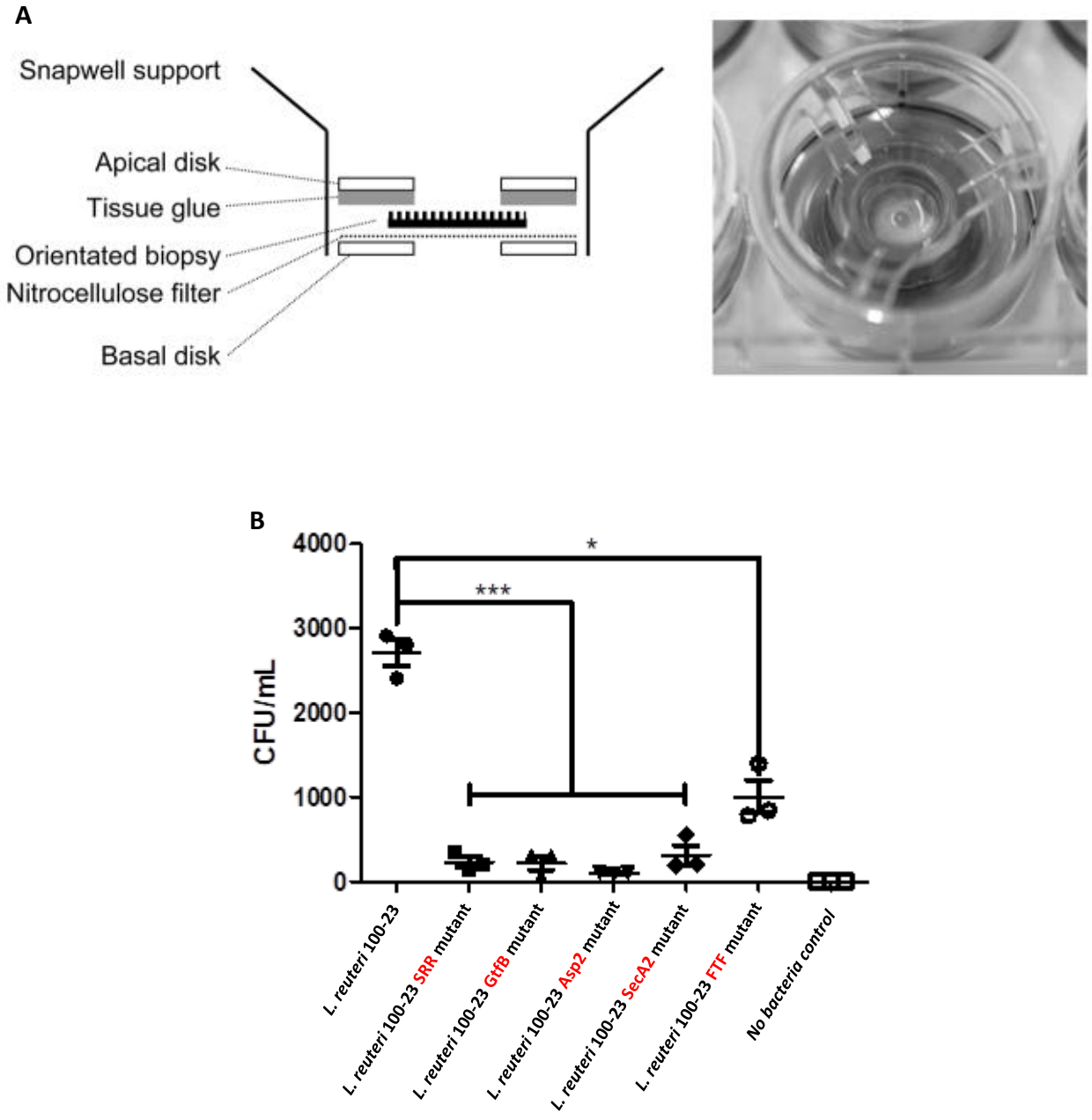

Figure 31: Adhesion of *L. reuteri* 100-23 and mutant strains to SPF murine forestomach.

PIVOC of forestomach was placed on the metal grid and 10^7 *L. reuteri* 100-23 wild-type or mutant strains were bacteria added to the cylinder in 25 µl DMEM for 1 h in a 5% CO_2 atmosphere at 37°C. At the end of the experiment, biopsies were removed from the Snapwell support, washed three times in PBS to remove non-adherent bacteria. After incubation for 1 h, cells were washed three times in PBS to remove non-adherent bacteria. Adherent bacteria were quantified by lysing cell monolayers in 1% Triton X-100 for 15 min and plating out serial dilutions of lysates on MRS agar plates. MRS plates were incubated at 37°C and colony forming units (CFU) were determined next day. Photo (A) taken from Schuller et. al., 2009

L. reuteri 100-23 WT strain showed reproducible binding to the murine forestomach whereas the mutant strains showed a significant decrease in adherence, suggesting that both the presence and glycosylation of the SRRP is crucial in the interaction of the rodent strain with the intestinal cells of the murine forestomach. The *ftf* mutant also showed reduced binding to the murine forestomach as compared to the WT strain (**Figure 31**), supporting the role of EPS in adhesion and biofilm formation.

4.3. Discussion

Adhesion to host tissues is an important first step of *L. reuteri* colonisation and is mediated by cell surface proteins[490]. Previous studies have demonstrated host adaptation of *L. reuteri* with evolution of host-specific binding proteins which enable rodent but not human, pig, or chicken isolates to colonise the gut of mice[31]. Here we showed that *L. reuteri* isolated from pig (*L. reuteri* ATCC 53608), human (*L. reuteri* ATCC PTA 6475) and rat (*L. reuteri* 100-23) varied in their ability to adhere to mucin or intestinal cell or tissue.

Here we showed that binding of *L. reuteri* strains to mucin was strain-dependent with *L. reuteri* ATCC 53608 showing the highest binding (compared to other strains tested) to purified porcine gastric mucin and that the binding was MUB-mediated. The role of MUB in *L. reuteri* ATCC 53608 adhesion to mucin was further confirmed under physiologically relevant shear conditions inducing a strong biofilm-forming phenotype. This may contribute to the ability to form biofilms under lateral flow, as demonstrated in our work. These results are line with previous studies showing strain-dependence binding of *L. reuteri* strains to mucus[388]. These cell surface molecules may have evolved due to adaptation of lactobacilli to their local environment[31]. Niche adaptation has previously been documented in *L. reuteri*, as genomic clades which contain adhesins that enhance adhesion to the host organism have been identified in strains isolated from humans, rodents, and pigs[376, 394].

We also showed that the presence of MUB on L. reuteri cell surface increased L. reuteri adherence to human-derived epithelial cells which do not produce mucins which supports previous work done that implicated MUB expressed on the porcine isolate ATCC 53608 in the interaction with human epithelial cells *in vitro*[491]. The ability of the ATCC 53608 to bind to human-derived epithelial cells might be due to similarities between the porcine and human GI tract[492]. it is important to note the ecological variation within the porcine and human GI tract however, with numerous *Lactobacillus* species forming biofilm layers in the pig GI tract[491]. Within humans, no such niche has been identified and relatively few *Lactobacillus* species are thought to be persistent in the GI tract[30]. Moreover, the ATCC 53608 strain

showed a more pronounced binding to mucin-expressing epithelial cells compared to the epithelial cells that do not produce mucins, indicating binding of MUB to both human epithelial cell surface molecules and mucins. The apparent mucin-binding property reported here for strains ATCC 53608 could reflect both the mucus recognition abilities and the propensity of this strain to auto aggregate, as previously reported using flow cytometry[388].

The human isolate *L. reuteri* PTA 5475 showed binding to the human-derived epithelial cells. In this interaction, the surface expression of CmbA on the human isolate *L. reuteri* PTA 6475 was shown to be involved in the interaction between the bacteria and epithelial cells. Our results, however, suggest that this adhesin does not contribute to the interaction of the *L. reuteri* ATCC PTA 6475 with either pPGM or mucin expressed on epithelial cells, which contrasted with earlier results that implicated the CmbA protein in the interaction with mucus[432, 433]. This might be due to the different types of mucin-producing epithelial cells used to assess the role of CmbA in the interaction of the bacteria to mucus. HT29-MTX used in this study consists almost exclusively of differentiated goblet cell-like phenotype secreting MUC1, MUC3, MUC4 and MUC5C predominantly expressed in the small and large intestine[487, 488]. In contrast, the goblet cell-like LS174T cell lines used in previous studies produce gel-forming mucins such as MUC1, MUC2, MUC5A/C and MUC6[493]. In view of this, intrinsic variations in mucin production by different cell lines need to be considered when addressing mechanisms of host-microbe interaction *in vitro*.

The rat isolate *L. reuteri* 100-23 showed binding to mouse forestomach but no binding to mucin or to the human colon cancer cell lines HT29 or HT29 MTX. This is line with the ecological niche of *L. reuteri* in rodents which is known to colonise the forestomach epithelium of mice and previous reports showing that mutant strains deficient in $SRRP_{100\text{-}23}$ and/or its dedicated transport system (the SecA2-SecY2 pathway) completely abrogated biofilm formation[378]. Here we used a polarized *ex vivo* model which allows directional access to the mucosal surface[489] and has been used as gold-standard to investigate pathogen infection such as enteropathogenic *E. coli*[432] or enterohaemorrhagic *E. coli*[494]. Using this model, we showed binding of *L. reuteri* 100-23 WT strain to the forestomach of mice compared whereas binding was impaired using *L. reuteri* SRRP and *aSec* mutants. This suggests that both the presence and glycosylation of the SRRP is crucial in the interaction of the rodent strain with cornified, stratified squamous epithelium which lines the proximal area of murine forestomach. The *ftf* mutant also showed reduced binding (as compared to the WT strain), albeit to a lower degree, indicating that EPS involvement in the interaction between the bacteria and mouse forestomach. Indeed, EPS produced

by *L. reuteri* DSM 20016 has been shown to be important for biofilm formation and adherence of the bacteria to epithelial surfaces[495]. Moreover, EPS biosynthesis has also been implicated in *in vitro* biofilm formation of *L. reuteri* 100-23[387]. Therefore, the initial adhesion facilitated here by SRRP, and EPS may represent the most significant step in biofilm formation, likely conferring host specificity.

The presence of diverse bile salts affects cell surface characteristics such as the properties of EPS, hydrophobicity, and rigidness, among other parameters, as well as adhesins[496, 497]. Here, we showed that the adhesion ability of *L. reuteri* ATCC 53608 strain to mucin was affected by bile salts to a greater extent than by acid treatment. This effect was not due to a decrease in cell viability as the percentage of survival of the ATCC 53608 strain was not affected following treatment by the simulated gastric conditions. Lactic acid bacteria employ various mechanisms in response to acid stress, including the maintenance of intracellular pH[498], preservation of cell membrane functionality[499] or induction of stress response proteins[500]. Proteomic analysis of *Lactobacillus casei* revealed multiple metabolic pathways which may be involved in the adaptation to acid stress including carbohydrate metabolism, signal transduction mechanisms, general functions, translation system, chaperones, nucleotide transport and metabolism, and amino acid biosynthesis[501]. The overexpression of proteins involved in carbohydrate metabolism plays an important role in some strains of *L. casei* by supplying energy to resist acid stress[502]. In addition, some enzymes involved in glycolysis were shown to be upregulated in the cell-surface fraction of *L. casei* grown at low pH, including enolase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase[503]. Interestingly these enzymes have been shown to be moonlighting proteins and implicated into adhesion of lactobacilli to mucin (**See section**). In addition, proteomic analysis of *Lactobacillus paracasei* 83123 revealed that proteins that were up regulated after GI stress were those involved in EPS biosynthesis[504]. It is therefore possible that the resistance of the ATCC 53608 exposure to external stress could be due to an increased EPS production and/or induction of cell-surface proteins which may in turn impact on the adhesion ability of the strains. The presence of bile has been shown to lead to decreased adhesion ability of *Lactobacillus delbrueckii* strains to epithelial cells, resulting in reduced persistence in the intestinal lumen and delayed capacity to activate the gut immune response[505, 506]. Bile salts have also been shown to decrease the adhesion of *bifidobacteria* to intestinal mucus[507]. Here, we hypothesised that the reduction in binding to mucus under bile stress observed in this study maybe due to the downregulation of cell-surface adhesins and/or EPS expressed by *L. reuteri* ATCC 53608. However, our results using western blot analysis, showed that $SRRP_{53608}$, but not MUB_{53608} expression was affected by bile stress, suggesting that other factors involved in bacterial adhesion and colonisation may contribute to the decreased bacterial

binding we observed *in vitro* between *L. reuteri* ATCC 53608 strain to mucin in the presence of bile salts. Long molecular dynamics simulations have been used previously to show that the binding region of $SRRP_{53608}$ adopts a right-handed parallel β-helical or "β-solenoid" fold not observed in other structurally characterised SRRPs and functions as an adhesin via a pH-dependent mechanism[508]. Therefore, it is also possible that the downregulation observed in this study following treatment with bile salts might be due to reduced ability of the antibodies used to detect the protein. Future work is warranted to determine the effect of bile salts on *L. reuteri* EPS production or hydrophobicity and Zeta potential[509, 510] and the impact of the treatment on *L. reuteri* binding to mucin.

Overall, we showed that cell surface-associated proteins play an important role in the adhesion of *L. reuteri* strains to different components of the GI tract. Such interactions might lead to the competitive exclusion of pathogens and/or the modulation of host cell responses. Moreover, given the diversity of the microbial population along the GI tract, it is expected that, *in vivo*, the expression and function of the lactobacilli adhesins is influenced by factors present in the dynamic environment of the GI tract (i.e., host responses, cell-to-cell communication molecules, etc). Therefore, unravelling the conditions that regulate the *in vivo* expression of these adhesins will contribute to a better understanding of *L. reuteri*–host interactions and influence on host health.

CHAPTER 5: ROLE OF BACTERIAL ADHESINS IN BINDING OF *L. REUTERI* STRAINS TO HOST LECTINS

5.1 Introduction

L. reuteri adhesins have been shown to be glycosylated. SRRP adhesins are differentially glycosylated in *L. reuteri* strains 100-23 and ATCC 53608, reflecting differences in the organisation of the SecA2/Y2 accessory cluster of these strains. *L reuteri* strains 100-23 (from rodent) and ATCC 53608 (from pig) can perform protein O-glycosylation and modify $SRRP_{100\text{-}23}$ and $SRRP_{53608}$ with Hex-Glc-GlcNAc and di-GlcNAc moieties, respectively, as shown by MS/GC-MS analyses[442]. Furthermore, *in vivo* glycoengineering in *E. coli* led to glycosylation of $SRRP_{53608}$ variants with α-GlcNAc and GlcNAcβ(1→6)GlcNAcα moieties as shown by NMR. This strain-specific glycosylation is reflected by the specificity of GtfC glycosyltransferase in SecA2/Y2 accessory secretion system with *L. reuteri* $GtfC_{53608}$ showing a preference for UDP-GlcNAc, and *L. reuteri* $GtfC_{100\text{-}23}$ for UDP-Glc, respectively, as demonstrated by DSF and STD NMR analyses[442]. Furthermore, preliminary data in the Group showed that MUB_{53608} binds *to Ricinus Communis* agglutinin (RCA), a β–Gal specific lectin, as demonstrated by force spectroscopy and further supported by RCA affinity chromatography, using competition assays with lactose[511]. Treatment of MUB_{53608} with α-galactosidase completely abolished its binding with RCA, further confirming the presence of terminal Gal moieties decorating the protein[511]. Differences in adhesin glycosylation profile may therefore contribute to the mechanisms underpinning *L. reuteri* adaptation to their host by recognising host specific lectins.

Lectins are cell surface glycoproteins that bind reversibly and nonenzymatically to specific carbohydrates[512-514]. Lectins act as recognition proteins, and by recognising complementary glycans structures (including monosaccharides, disaccharides, and polysaccharides) on other cells, they mediate both cell-to-cell and cell-to-molecule interactions[515]. This protein-glycan interaction occurs through the carbohydrate recognition domain (CRD) of lectins. This type of interaction is mostly specific but occurs with low affinity and requires multivalency derived from both lectins and carbohydrate antigens to achieve avidity of binding[516]. The biological functions of lectins in micro-organisms and animals are diverse, and include roles in infection defence, innate immunity, glycoprotein synthesis, and cell cycle regulation[516, 517]. Host lectins are mainly expressed by immune cells, but some are also produced by epithelial cells[518, 519]. They can be secreted or bound to the cell membrane (transmembrane proteins). Here, we tested the ability of *L. reuteri* glycosylated adhesins to bind to host lectins in the gut which may play a role in gut microbe-host interactions are the mucosal surface.

Galectin-3 (Gal-3) is a unique β-galactoside-binding lectin and is one of the most ubiquitously expressed members of the galectin family, demonstrating elevated expression levels in the epithelial cells of the digestive tract[159]. Gal-3 has pleiotropic biological functions such as influencing cell growth, cell adhesion, cell-cell interaction, and as a pre-mRNA splicing factor[159]. Within the intestinal tract, Gal-3 is detected predominantly in the villus tips[520] and in the mucus layer[521] and interacts with MUC2, a secreted mucin found in the intestine[522, 523]. Extracellular Gal-3 has been shown to interact with a range of pathogenic bacteria including *Neisseria gonorrhoeae*[524], *C. albicans*[525], *Schistosoma mansoni*[526] *and Trypanosoma cruzi*[527, 528], and further regulate innate immune response. Using an indirect surface plasmon resonance-based approach, Gal-3 was also shown to interact with commensal bacteria (two strains of *Bifidobacterium longum* subsp. *Infantis)*[171].

The human intelectin-1 (hINTL-1) is a Ca^{2+}-dependent D- galactofuranose-specific lectin which can discriminate between microbial and human glycan epitopes through a protein-bound calcium ion-dependent coordination of a terminal, acyclic 1,2-diol, which is a component of microbial monosaccharides[177]. D- galactofuranose is often found in fungal cell walls and glycoproteins[529] as well as in carbohydrate structures from important human parasites and bacterial pathogens[530]. Previous work in the Group reported that *L. reuteri* ATCC 53608 encodes a gene encoding a predicted galactopyranose mutase, an enzyme that can convert UDP-Gal to UDP-galactofuranose (UDP-Galf)[511].

In this chapter, we tested the role of *L. reuteri* ATCC53608 and 100-23 adhesins, namely MUB_{53608} and $SRRP_{100\text{-}23}$, respectively, in mediating the interaction with Gal-3 and hINTL-1 lectins.

5.2 Results

5.2.1 Purification of MUB and SRRP from L. reuteri ATCC 53608 and 100-23 strains

In order to directly assess the role of *L. reuteri* adhesins in the interaction of the bacteria with intestinal lectins, native MUB and SRRP glycosylated proteins were purified from *L. reuteri* 53608 (1063N) and 100-23, respectively. MUB was purified from large-scale cultures of *L. reuteri* 1063N grown in LDM II by gel permeation chromatography (GPC) (see Materials and Methods), at an estimated yield of 1mg/mL. *L. reuteri* 1063N was preferred to *L. reuteri* ATCC 53608 strains for MUB purification as this natural variant lacks the LPxTG cell wall anchor motif and thus the ability to attach to the cell surface[388], therefore most of truncated MUB (tMUB) is released in the supernatant, facilitating the purification process. Truncated MUB comprises Mub repeats RI, RII, RIII, RIV, R1, and part of R2, compared with the 14 Mub repeats in the full-length MUB but retains its glycosylation profile. Here, the purified protein was detected on western blotting using a FITC-labelled lectin, f-RCA, Gal or N-acetylglucosamine (GalNAc) specific lectin. Purified tMUB showed an apparent mass of ~400 kDa in an SDS-PAGE (**Figure 32**) which is in agreement with the presence of glycosylation. The yield of purification of 9 mg/ml.

Based on its glycosylation profile, $SRRP_{100\text{-}23}$ was purified by affinity chromatography, using an agarose-bound Wheat Germ Agglutinin (WGA) column and bound proteins were eluted with GlcNAc (see Materials and Methods). The collected fractions were analysed by SDS-PAGE (**Figure 32**). The purified $SRRP_{100\text{-}23}$ was detected on western blotting using a FITC-labelled lectin, f-WGA lectin recognising β-1,4-GlcNAc-linked residues[531]. The major purified protein migrated at an apparent molecular weight >300 kDa.

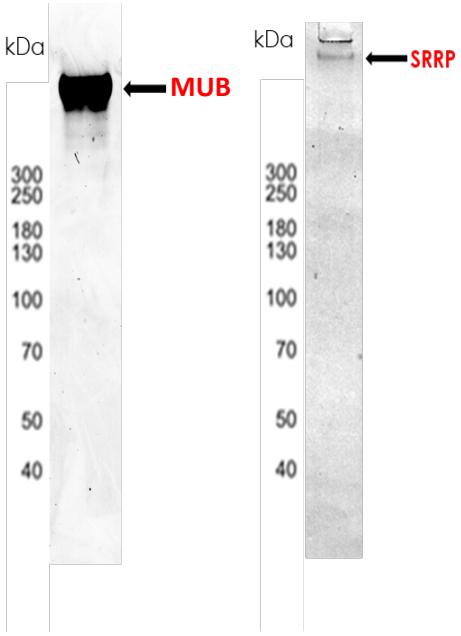

Figure 32: Western blot detection of the 1063N MUB (A) and SRRP100-23 (B) adhesins.

Proteins were separated on an 4-12% SDS-polyacrylamide gel, transferred to PVDF membrane by electroblotting, and probed with f-RCA (A) or f-WGA (B).

To confirm the glycosylation profile of the purified protein $SRRP_{100\text{-}23}$, the nature of monosaccharides decorating this adhesin was determined by gas chromatography-mass spectrometry (GC-MS) following methanolysis, N-acetylation and TMS-derivatisation of the released methyl-glycosides (see Materials and Methods).

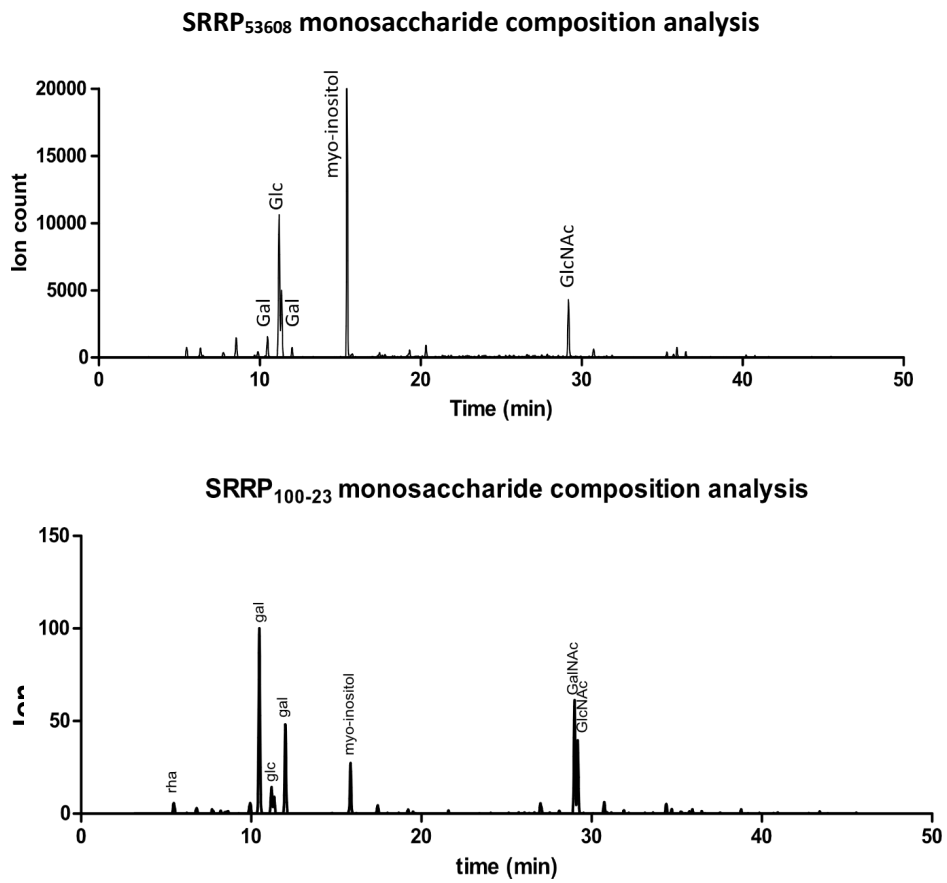

Figure 33: GC-MS chromatogram of monosaccharide composition analysis of $SRRP_{100-23}$ and $SRRP_{53608}$.

The analysis showed the presence of Glc, Gal and GlcNAc in both strains, in addition to Rha in *L. reuteri* ATCC 53608. Myo-inositol as used as internal standard.

The results showed the presence of Gal, as well as smaller amounts of Glc, GalNAc, GlcNAc and Rha in $SRRP_{100\text{-}23}$ (**Figure 33**). These results are consistent with previous MALDI-ToF analysis of $SRRP_{100\text{-}23}$ which indicated that the Hex and HexNAc ratio would be found in a 2:1 ratio. Concurrent with that, the total amount of Hex molecules (Glc and Gal) was found twice higher than GlcNAc (**Figure 33**). However, the presence of rhamnose (Rha) was not expected as no deoxy-hexose had been identified in the MALDI-ToF analysis of $SRRP_{100\text{-}23}$. It may be that only a small fraction of the glycans carries a Rha residue, which will be below the detection limits of MALDI-ToF, under the conditions tested in the original study[442]. Together, these data indicate that $SRRP_{100\text{-}23}$ is glycosylated with Gal-Glc-GalNAc-GlcNAc sugar moieties. Note that the same approach was applied to $SRRP_{53608}$ which is produced by *L. reuteri* 53608. Here we showed that $SRRP_{53608}$ is glycosylated with GlcNAc-GlcNAc sugar moieties.

Due to its extensive glycosylation profile, we weren't able to quantify purified SRRPs which hampered our efforts to assess the role of purified SRRPs in their interaction between the bacteria and host lectins. In the following sections, we focused on the role of MUB in the interaction between *L. reuteri* ATCC 53608 and intestinal lectins. The role of the surface expression of $SRRP_{100\text{-}23}$ and its glycosylation in the interaction between *L. reuteri* 100-23 and immune lectins will be assessed in the next chapter using the *L. reuteri* 100-23 WT and mutant strains.

5.3. Binding of MUB to host lectins

5.3.1 MUB binds to hINTL-1

As *L. reuteri* ATCC 53608 harbours genes for the synthesis of UDP- Galf, a known ligand of hINTL-1, and MUB_{53608} was shown to contain Gal residues, hINTL-1 was tested for its ability to bind MUB. The interaction between MUB and hINTL-1 was first tested by western blot following electrophoresis and transfer of MUB onto a membrane and probing with biotinylated hINTL-1 or fluorescently labelled RCA. StrepMAB-Classic-HRP conjugate antibodies were used for the detection of hINTL-1.

Using this approach, no interaction was observed between hINTL-1 and MUB (**Figure 34A)** whereas MUB was recognised by f-RCA used as a positive control (**Figure 34B**). Prior to electrophoresis on SDS-PAGE and western blot analysis, the protein sample is heated to denature the higher order structure to ensure that the negative charge of amino acids is not neutralised, enabling the protein to move into an electric field. This denaturation step may affect the conformation and glycan-lectin interaction.

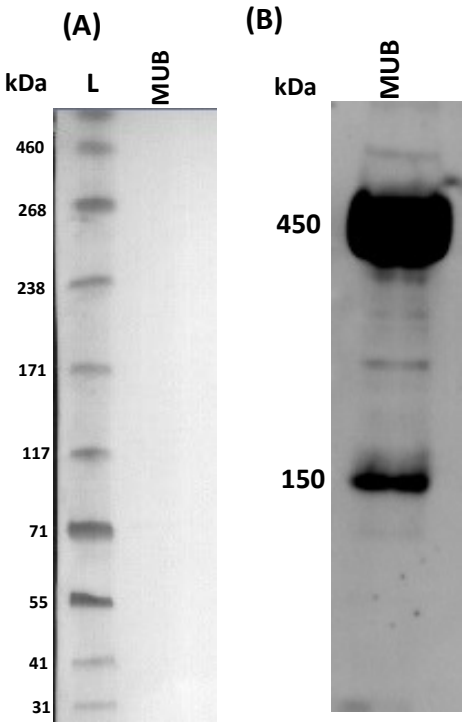

Figure 34: Western blot analysis of the interaction between hINTL-1 and MUB.

Samples were separated by electrophoresis on an 4-12% SDS-PAGE, transferred to a PVDF membrane by electroblotting, and probed with **(A)** hITLN1 in the presence of Ca^{2+} or **(B)** RCA.

To test whether these proteins could interact in their native state, a slot blot was performed by blotting hINTL-1 onto the membrane containing MUB and BSA as a negative control. The use of RCA and anti-MubR5 confirmed the presence of MUB (**Figure 35C**). The slot blot analysis with hINTL-1 showed that hINTL-1 and MUB were able to interact (**Figure 35**) and that this interaction only occurred in the presence of Ca^{2+} (**Figure 35A**). In addition, the interaction was abolished by EDTA, confirming the specificity of the interaction (**Figure 35B**).

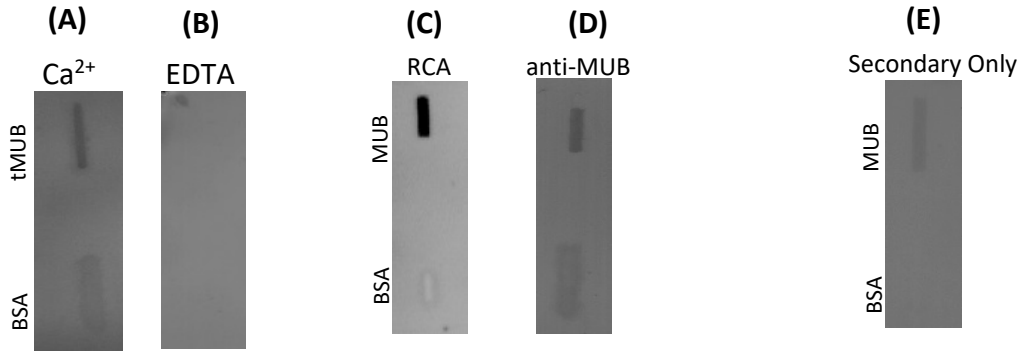

Figure 35: Slot blot analysis of the hINTL-1 interaction with MUB.

In the presence of Ca^{2+} **(A)**, following addition of EDTA **(B)** or in the presence of the secondary antibody only **(E)** only. f-RCA **(C)** and anti-MUB antibody **(D)** were used as positive controls.

However, it was noted that the secondary antibodies (anti-rabbit) used as control could directly recognise MUB (**Figure 35E**), which may be due to the presence of Ig-binding protein domains in the Mub repeats constituting the protein[429]. Therefore, to confirm that the between MUB and hINTL-1 was specific, we used atomic force spectroscopy. Interaction of MUB and hINTL-1 resulted in adhesion events ranging from 100 to 500 pN (**Figure 36, green line**). When EGTA was added to the mixture, the frequency of the adhesion events decreased (**Figure 36, purple line**), confirming the specificity of the interaction.

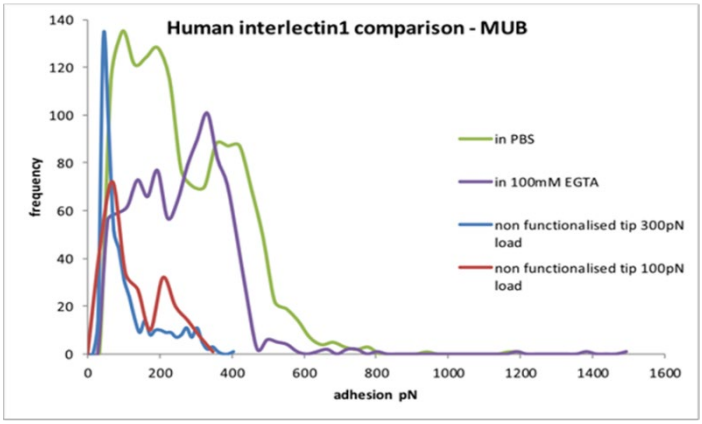

Figure 36: Force Microscopy analysis of the hINTL-1 interaction with MUB.

Force spectroscopy curves, showing the interaction of MUB with hINTL-1 in PBS with Ca^{2+} **(green line)** or in the presence of EGTA **(purple line)**. Non functionalised tip 100pN **(red line)** and non-functionalised tip 300pN **(blue line)** were used as negative controls.

5.3.2 MUB binds to Gal-3.

As shown above using slot blot and western blot, RCA can recognise MUB, due to the presence of Gal or GalNAc decorating the protein (**section**). Here, the interaction between MUB and human recombinant Gal-3 was investigated by ELISA using recombinant His-tagged Gal-3 expressed in *E. coli* and purified using IMAC and affinity chromatography lactose column (**see Materials and Methods**). RCA and BSA were used as positive and negative control, respectively (**Figure 37**). Purified MUB was able to bind to RCA or Gal-3 in a significant manner as compared to BSA (**Figure 37**).

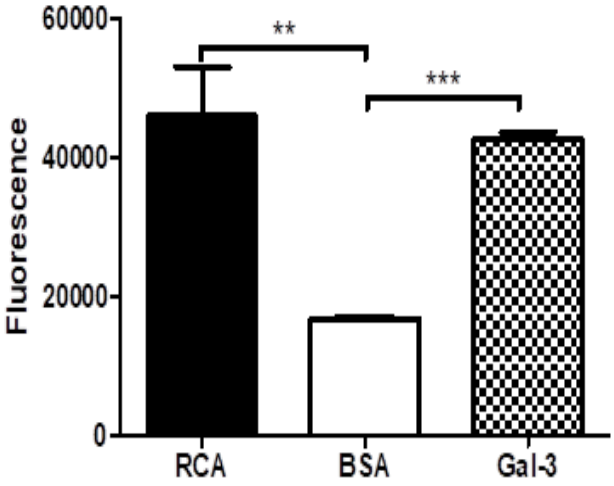

Figure 37: Binding of recombinant Gal-3 to MUB.

MUB was added to either Gal-3, RCA (positive control) or BSA (negative control) coated plates. Following washing, binding was detected using primary anti-MubR5 or anti-MubRI antibodies (1: 10000) and secondary antibody anti-rabbit IgG alkaline phosphatase (AP) (1:10 000). Substrate pNPP (1 mg/mL pNPP in 0.2 M Tris 5 mM $MgCl_2$, pH 9.6-10.5) was added to the wells and absorbance was measured at 405 nm. Mean values ± SD are shown. Statistical differences were analysed by the Student's t-test, with significance defined as ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001, and ∗∗∗∗P < 0.0001.

The specificity of the interaction was further tested by determining MUB ability to inhibit the interaction between Gal-3 and asialofetuin (ASF), a β-galactose-specific glycoprotein and known a ligand of Gal-3 using ELISA (**Figure 38**). Lactose (a well-characterised ligand Gal-3) and BSA were also included as positive and negative control, respectively. Gal-3 binding to ASF in the absence of BSA, lactose or MUB was used to define the 0% inhibition response. Lactose and purified MUB could significantly inhibit the interaction between Gal-3 and ASF (**Figure 38**), showing 69.7% and 42.16% ($P < 0.001$) inhibition, respectively.

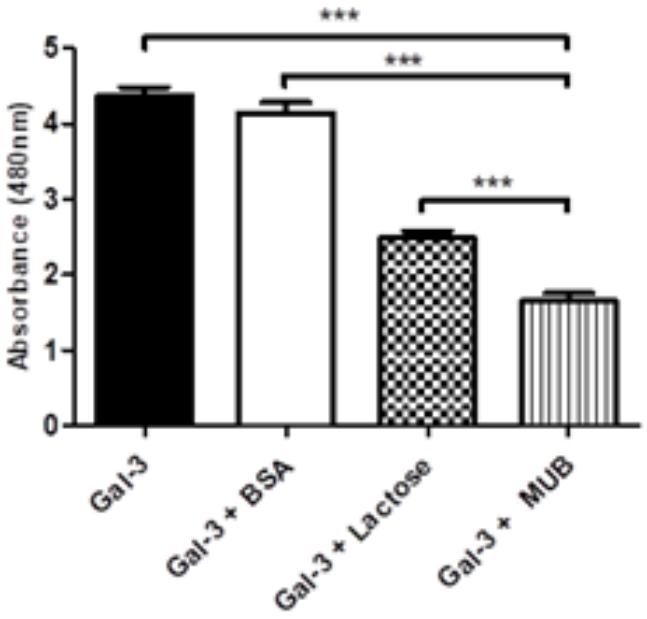

Figure 38: Inhibition of Gal-3 interaction to immobilised ASF by free MUB.

Gal-3 on its own or together with BSA (negative control), lactose (positive control) or MUB was added to ASF-coated plates. Following washing, binding was detected using a primary anti-his tag antibody (1: 10000) and secondary antibody anti-APC (1: 10 000), and the absorbance was measured at 405 nm. Each assay was performed in triplicate and the data were analysed in Excel (Microsoft, Washington, USA). Mean values ± SD are shown. Statistical differences were analysed by the Student's t-test, with significance defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

5.4. MUB mediates the interaction between Gal-3 and *L. reuteri* ATCC 53608

To determine the role of MUB in the interaction of Gal-3 to the bacteria, the ELISA binding assay using high-binding polystyrene microtitre plate wells coated with Gal-3, BSA (negative control) or RCA (positive control) was carried out with cFDA labelled *L. reuteri* ATCC 53608 (**Materials and Methods section**). The values were expressed as percentage binding values normalised to the mean value for strain *L. reuteri* ATCC 53608, used as an internal control on the plate. Gal-3 was found to significantly bind to *L. reuteri* ATCC 53608 with values comparable to those obtained when RCA was used as a positive control (**Figure 39**). In addition, the binding between the bacteria and Gal-3 was significantly reduced, although not abolished, when the binding was performed in the presence of lactose (**Figure 39**), which suggests the occurrence of additional interactions with the bacteria.

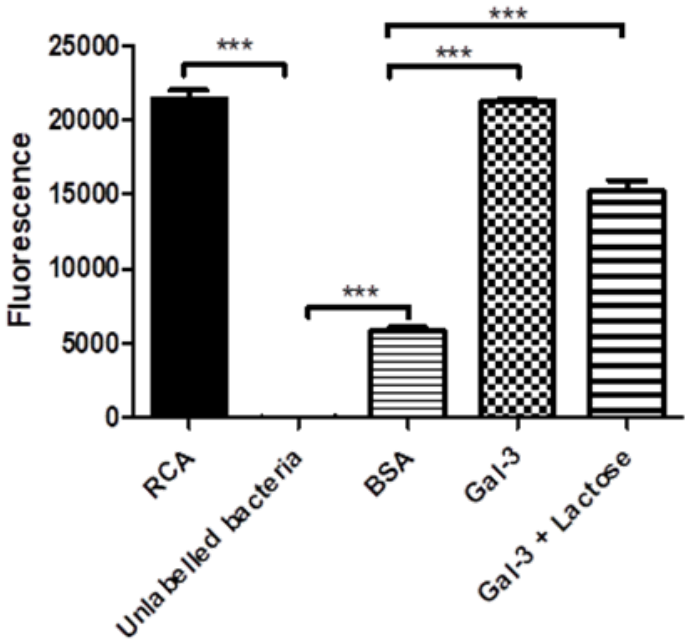

Figure 39: Binding of *L. reuteri* ATCC 53608 strain to recombinant Gal-3.

L. reuteri ATCC 53608 bacteria grown to early stationary phase and cF-labelled were added to Gal-3, Gal-3 (preincubated with lactose) or BSA coated plate. Following washing, bound bacteria were lysed with SDS in NaOH. The released cF fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 485 and 520 nm as excitation and emission wavelengths, respectively. Each assay was performed in triplicate. Standards were included on each occasion also in triplicate consisting of SDS lysed cF-labelled bacteria to evaluate the % adhesion. Mean values ± SD are shown. Statistical differences were analysed by Student's t-test, with significance defined as ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001, and ∗∗∗∗P < 0.0001.

Next, we determined whether binding of Gal-3 was dependent on the presence of MUB on the bacterial surface. Flow cytometry was used to study the binding of f-RCA or Gal-3 to *L. reuteri* ATCC 53608 (expressing MUB on the cell surface) or *L. reuteri* 1063N grown in LDM II media. There was a strong interaction between Gal-3 or RCA (used as a positive control) and *L. reuteri* ATCC 53608 (**Figure 40E and Figure 40F**). The MUB mutant, 1063N strain, however, showed no ability to bind to Gal-3 or to RCA, irrespective of the concentration of the proteins used, indicating that MUB is an important contributor of the interaction.

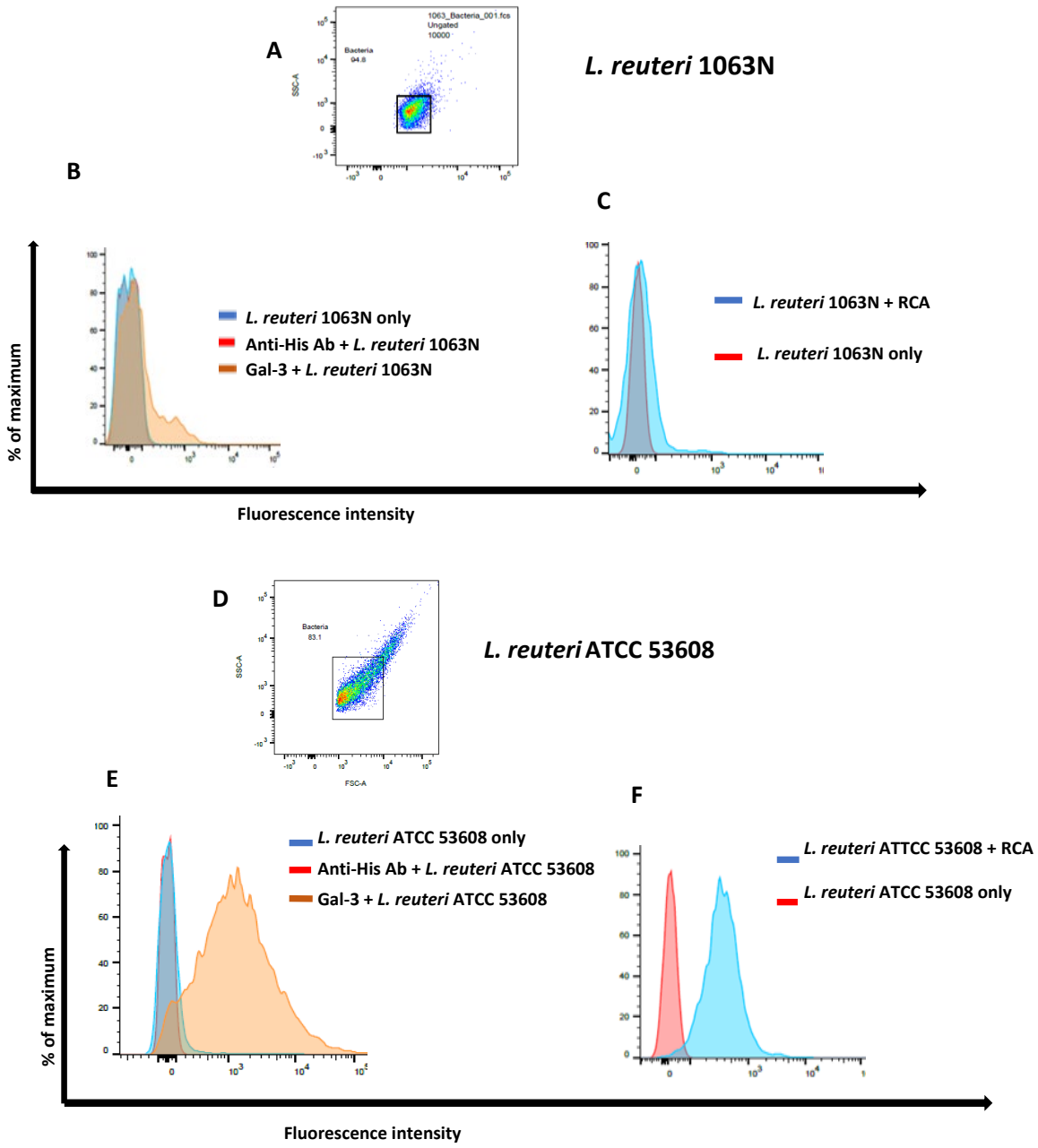

Figure 40: MUB mediates the interaction between Gal-3 and *L. reuteri* ATCC 53608 or RCA.

A total of 2.5x10^7 bacteria, grown in MRS were incubated with purified His-tagged Gal-3 or f-RCA. Following washing, the interaction to Gal-3 was detected using primary anti-His-tag antibody (1:5000) and anti allophycocyani (APC) secondary antibody (1:2000). **(A)** *L. reuteri* 1063N gating strategy **(B)** binding assay of *L. reuteri* 1063N and Gal-3 **(C)** binding assay of *L. reuteri* 1063N and RCA **(D)** *L. reuteri* ATCC 53608 gating strategy **(E)** binding assay of *L. reuteri* ATCC 53608 and Gal-3 **(F)** binding assay of *L. reuteri* ATCC 53608 and RCA. Fluorescence intensities were measured by FACS Calibur (BDBiosciences, USA). The data were analysed using the FlowJo software version 5.7.1 (Tree Star, USA).

It is worth noting that *L. reuteri* ATCC 53608 strain appeared to form multicellular aggregates (**Figure 40A and Figure 40D**), in line with previous work suggesting that MUB contributed to the ability of *L. reuteri* ATCC 53608 to auto aggregate using flow cytometry or form biofilms using bioflux (**section 4.2.1.2**).

5.5 Discussion

Protein glycosylation is emerging as a crucial property across bacteria species. Protein glycosylation systems have been reported and studied in many pathogenic bacteria, revealing an important diversity of glycan structures and pathways within and between bacterial species[532]. However, protein glycosylation systems are also found in commensal bacteria and therefore no longer exclusively associated with a canonical virulence factor as defined by the criteria established by Falkow[533]. Nonetheless, the nature and function of protein glycosylation in gut commensal bacteria remains largely unexplored[534].

Here, we first confirmed previous findings that that the rodent isolate *L. reuteri* strain 100-23 can perform protein *O*-glycosylation and modify $SRRP_{100\text{-}23}$ with Gal-Glc-GalNAc-GlcNAc. We previously showed (Chapter 4) that $SRRP_{100\text{-}23}$, contributed to the binding of *L. reuteri* 100-23 to the forestomach epithelium. Although, the interaction is to occur via the binding region (BR) of SRRP to host epithelial cells and DNA[165], it is possible that protein-glycan interactions may also contribute to the binding conferring a level of host specificity. Indeed BR-SRPPs are conserved between *L. reuteri* strains recognising different hosts[165], suggesting that other factors may contribute to the host-microbe interaction. BR-$SRRP_{53608}$ has been shown to recognise components of the ECM as well as mucins[508]. In the future, it will be interesting to assay the binding of SRRP to lectins found on the epithelium or in the mucus as done for MUB in this chapter. We showed that purified MUB was recognised by RCA, a Gal specific lectin, also in line with its high apparent molecular weight. We confirmed that the interaction with RCA was glycan-mediated by force spectroscopy and RCA affinity chromatography, using competition assays with lactose. The interaction with RCA was previously reported for other secreted proteins from *L. reuteri* ATCC 53608[511], suggesting that this adhesin may be a target protein of a general glycosylation system.

Next, the role of the *L. reuteri* ATCC 53608 surface adhesin, MUB, was assessed for its binding specificity to host lectins. Using slot blot and force microscopy analysis, we demonstrated that tMUB purified from L. *reuteri* 1063N could bind to recombinant hINTL-1. hINTL-1 is a Ca^{2+} -dependent secreted lectin,

expressed in intestinal goblet and Paneth cells[535], able to distinguish microbial from host cells, as it recognises carbohydrates not naturally found in mammals[145, 178]. The binding of hINTL-1 to MUB was reduced (but not completely abrogated) in the presence of a chelating agent which suggests that the interaction is mainly mediated by glycans found on the protein, but that there is also protein-protein interaction, albeit to a lesser degree. The recognition of bacteria by intelectins is crucial as they are involved in physiological and pathological processes such as immune defence induced by bacterial infection, PAMP recognition, T helper cell type 2 immune responses induced by parasites, asthma, iron metabolism, obesity and cancer[145]. For example, hITLN-1 has been shown to specifically recognise to Bacillus Calmette-Guérin (BCG), which acts as an opsin to facilitate BCG phagocytosis[179]. In addition, exogenous mice ITLN-1 was also observed to enhance the phagocytosis of BCG by immune cells suggesting that intelectin may be a host defence lectin that assists phagocytic clearance of micro-organisms[179]. At the structural level, it has been reported that recombinant hITLN-1 protein expressed in RK-13 cells in the presence of calcium can specifically recognise to ribofuranose containing furan residues (Ribf) and disaccharide containing β-galactofuranose residues (derived from *Mycobacterium* species and the cell wall of *Nocardia* species), suggesting that hITLN-1 participates in the pattern recognition induced by galactofuranose[177, 536]. Therefore, the specific recognition of galactofuranose residues that may decorate the bacterial surface of *L. reuteri* ATCC 53608 strain by the lectin may allow the bacteria to interact with the host's immune system as galactofuranose residues are widely present in microbes but not in human glycans[537-540]. Several attempts including mutating predicted genes involved in the formation of galactofuranose and sugar nucleotide analysis were made to detect the presence of galactofuranose on Mub. However, these experiments were not successful. It is possible that Mub is decorated with very few Gal residues which resulted in values below detection limit in the process of identifying Gal in furanosic configurations. In the future, it will be interesting to employ glycosidic linkage analysis to assess if the Gal residues on Mub we identified in this study are present in furanosic configurations.

We also showed that both purified and surface expressed MUB plays an important role in the interaction between the *L. reuteri* ATCC 53608 strain and Gal-3. The specificity of the interaction was further highlighted as purified MUB was able to inhibit the interaction between Gal-3 and ASF, a β-galactose-specific glycoprotein and known a ligand of Gal-3[541]. Gal-3 is expressed in many cell types and participates in a broad range of physiologic and pathologic processes, such as cell adhesion, cell activation, chemoattraction, cell cycle, apoptosis, and cell growth and differentiation[542]. Several studies have also shown Gal-3 to interact with different mucins, including the ocular cell surface MUC, MUC1

and -16, in a galactose-dependent manner[543] or to cancer mucins from human colon cancer cell lines[523]. Previous *in vitro*, cell-based, and force spectroscopy assays in our lab has also demonstrated that mucins could directly interact with Gal-3[544]. The glycan-lectin interaction between galactose moieties on the MUB and Gal-3 observed here may define how *L. reuteri* ATCC 53608 colonise the host epithelial cells and mucus layer of the GI tract. Indeed, Gal-3 is a major component of intestinal mucus layer, as shown by proteomics of mucus in GI tract of mice[545].

The ability of *L. reuteri* ATCC 53608 strain to interact with these intestinal lectins *in vitro* may suggest a role for MUB in mediating *L. reuteri* host immune response *in vivo*. In a healthy state, MUB-mediated bacteria binding to Gal-3 or hINTL-1 may ensure that *L. reuteri* remains confined to the outer mucus layer which is known to contain secreted these lectins[546], whereas, in diseased states where the gut barrier is compromised, the MUB-mediated bacteria binding to Gal-3 or hINTL-1 expressed at the surface of epithelium cells may provide an additional layer of immunity and protection.

CHAPTER 6: ROLE OF *L. REUTERI* CELL SURFACE ADHESINS ON *L. REUTERI* IMMUNOMODULATORY PROPERTIES

6.1 Introduction

L. reuteri strains have been shown to be beneficial to the host through modulation of the gut microbiome[547-549], reinforcement of the intestinal barrier[550, 551], and interaction with the innate and adaptive immune system[552, 553]. Competitive exclusion of pathogens either via secretion of antimicrobial compounds or via inhibition of pathogenic adhesion is another mechanism generally associated to *L. reuteri*[386, 554, 555] **(see section 2.3.2).** However, little is known on the molecular receptors involved in *L. reuteri* immunomodulatory properties. Here we explored the role of glycosylated cell surface adhesins in the interaction between *L. reuteri* strains and immune cells.

Dendritic cells (DCs) are migratory phagocytic cells that act as the gatekeepers of the immune system[556, 557]. Tissue resident DCs in the GI tract mediate tolerance to commensal microbes and food antigens, while facilitating the appropriate response to pathogens[558]. The specific properties of DCs are influenced by both host and microbial signals[558]. Intestinal DCs are known to sample the gut microenvironment via antigen uptake and respond to environmental cues. The ability of DCs to regulate intestinal homeostasis mostly depends on their maturation[556, 559, 560]. Immature DCs are observed to be assembled at sites of inflammation following microbe internalisation and then migrate to T cell-rich areas within lymphoid organs following gaining the appropriate stimuli[561]. In the lymphoid organs, DCs undergo maturation and modulate their cytokine and cell surface expression profiles. For example, stimulated mature DCs express high levels of CD40, CD80 and CD86 which in turn induce activation of T cells[562]. The bridge between stimulation and transcription is linked by a complex network of signalling pathways that work to activate transcription factors such as nuclear factor-κB (NF-κB), which translocates to the nucleus and initiate discrete transcriptional programs[563]. This translocation occurs when DCs initiate an inflammation process upon external stimuli, resulting in the production of cytokines, which are the key players in inflammation. NF-κB is crucial for a multitude of important immunological transcriptional processes, including inflammatory responses microbes by immune cells[564, 565], development and activation of adaptive immune cells[566], as well as the development of secondary lymphoid organs[567]. Multiple *in vitro* studies have demonstrated that *L. reuteri* can induce anti-inflammatory Treg cells and suppress Th1/Th2 responses[387, 445, 568-571]; effects which are suggested to contribute to the beneficial influence of *L. reuteri*

on the host. However, the detailed molecular mechanisms by which *L. reuteri* interacts with DCs to modulate immune responses and promote gut homeostasis remain underexplored.

Membrane vesicles have been recognised as a form of cell-cell communication used by almost all domains of life: bacteria, archaea and eukaryotes. Much of the understanding of the role of bacterial extracellular vesicles (BEVs) mediating intercellular signalling is derived from studies of pathogenic bacterial outer membrane vesicles, showing that they are involved in the pathogenesis via delivering virulence factors to target cells[572, 573]. In recent years, there has been an increasing interest in the role played by BEVs produced by probiotic and commensal microbes in their communication with the host[409]. *L. reuteri* strains DSM 17938 and ATCC 23272 have been shown to produce BEVs[409, 574]. In these studies, the regulatory actions of *L. reuteri*-derived BEVs was shown to be important in the maintenance of intestinal immune homeostasis both *in vivo* and *in vitro* experiments. Here, we hypothesised that these BEVs may contain cell-surface adhesins contributing to the modulation of the host immune response.

In this chapter, we studied the role of *L. reuteri* adhesins present on the cell surface of bacteria or BEVs on the modulation of immune response by human monocyte-derived DCs (moDCs) and mice bone marrow-derived DCs (BMDCs) *in vitro*.

6.2. The role of *L. reuteri* cell surface components on the immunomodulation of DCs

6.2.1 The role of L. reuteri strains in cytokine production by BMDCs

In order to optimise, the conditions of the *in vitro* assays, we first investigated the effect of increasing bacterial concentrations (multiplicity of infection, MOI, from x1 to 500) of *L. reuteri* 100-23 strain on TNF-α production by BMDCs using ELISA. The results showed a strong *L. reuteri* dose-dependent increase in the production of TNF-α as compared to untreated BMDCs (**Figure 41**).

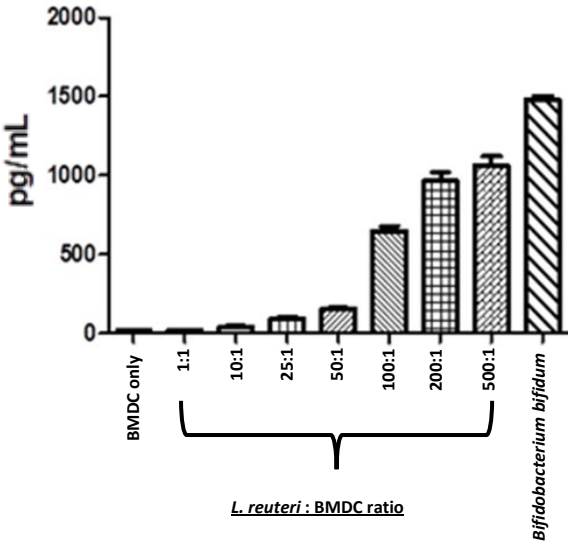

Figure 41: *L. reuteri* dose-dependent response of TNF-α production in mouse BMDCs.

BMDCs were co-incubated with *L. reuteri* 100-23 or with *Bifidobacterium bifidum* as a positive control, for 18 h. Concentrations of bacteria ranged from 5.0 × 10^5 to 2.5 × 10^8 CFU/mL (MOI, 1 to 500). The concentration of TNF-α cytokine (pg/mL) was then measured by ELISA. Mean values ± SD are shown.

Although TNF-α could not be detected in untreated BMDCs or in *L. reuteri* 100-23-treated BMDCs at MOI of 1 and 10, 210pg/mL of TNF-α was detected at MOI of 25, reaching > 1000 pg/mL at MOI of 500 which are comparable to levels obtained following *Bifidobacterium bifidum* induction at MOI of 50 (used as a control). The observation that, at high doses, *L. reuteri* strains (or *B. bifidum* strain) can have the ability to induce elevated levels of proinflammatory cytokines such as TNF-α *in vitro* maybe relevant in the context of a compromised gut barrier where commensal bacteria may make direct contact with immune cells.

In the rest of the study, all co-culture experiments were performed with *L. reuteri* strains at MOI of 100 as it is the lowest MOI showing a significant difference in cytokine production by BMDCs induced by the *L. reuteri* 100-23 strain.

To determine the role of *L. reuteri* cell-surfaced adhesins in the ability of the strains to induce an immune response *in vitro*, we first tested *L. reuteri* 100-23 *aSec* mutants, *SecA2, Asp2, GtfB*, as well as insertion mutants targeting cell surface proteins SRRP and LSP. In addition, since elimination of *L. reuteri* 100-23 EPS due to a mutation of the fructosyl transferase (*ftf*) gene has been shown to affect the bacteria's ability to induce an immune response, we also included *L. reuteri* 100-23 *ftf* mutant in our study.

Following incubation with *L. reuteri* strains, pro-inflammatory (TNF-α) and anti-inflammatory cytokines (IL-10) concentrations were determined in supernatants of BMDC cultures by ELISA.

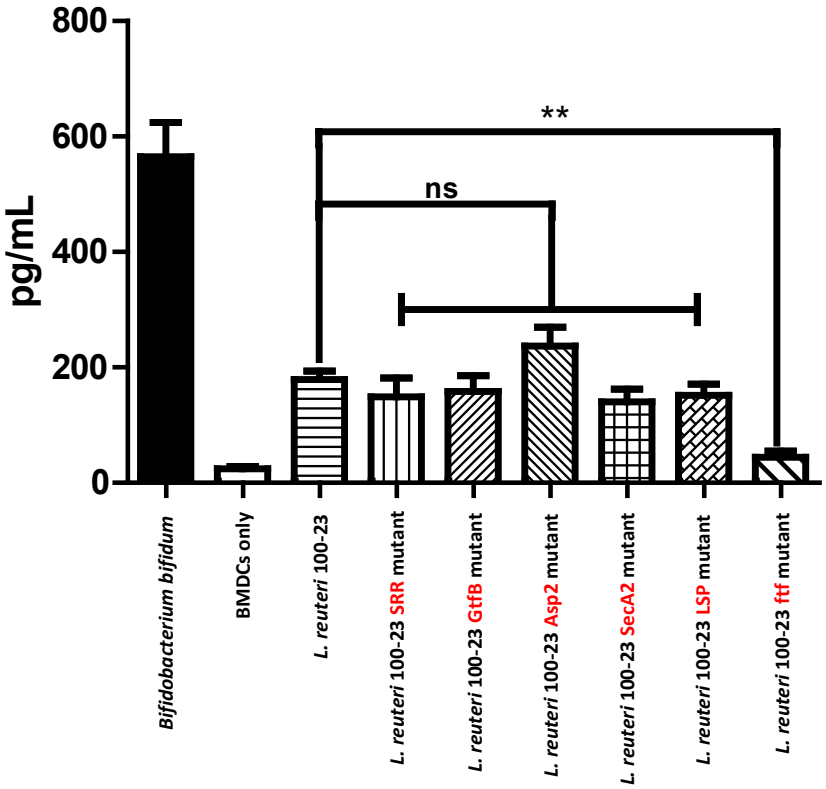

Figure 42: Effect of *L. reuteri* 100-23 strains on IL-10 production by BMDCs.

Mouse BMDCs were co-incubated with *L. reuteri* 100-23 WT and mutant strains, or with *Bifidobacterium bifidum* as a control, for 24 h. The concentration of IL-10 cytokine was measured by ELISA.

L. reuteri strain 100-23 WT and its mutants induced the production of anti-inflammatory cytokine IL-10 as compared to the unstimulated BMDCs cells (**Figure 42**). However, no difference in IL-10 production was observed between the *L. reuteri* 100-23 aSec and *LSP* mutants, as compared to the WT strain. The *ftf* mutant, however, induced reduced production of IL-10 compared to the WT strain suggesting that EPS may play a role in preventing inflammation.

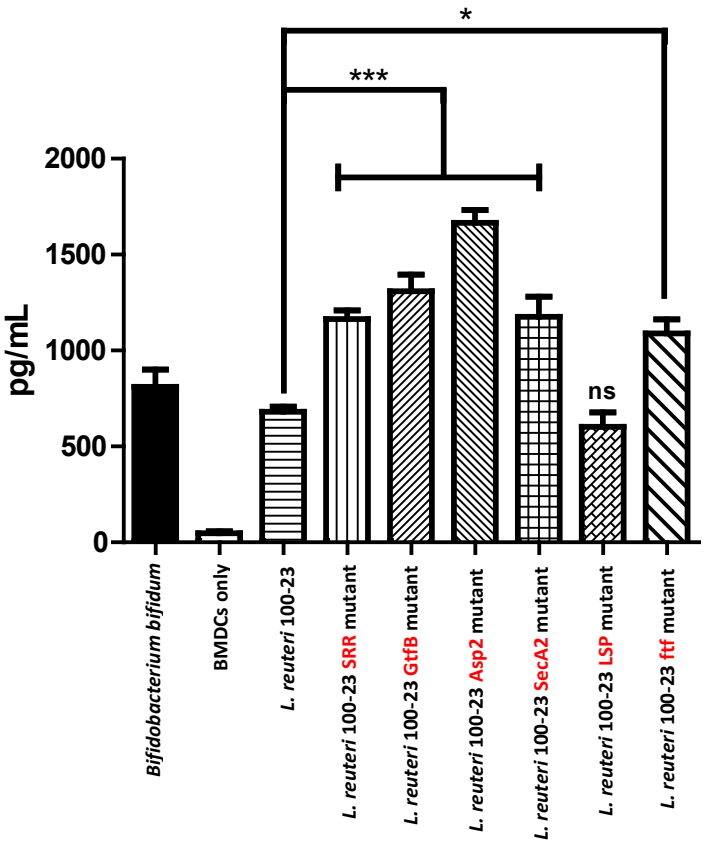

Figure 43: Effect of *L. reuteri* 100-23 strains on TNF-α by BMDCs.

Mouse BMDCs were co-incubated with *L. reuteri* 100-23 WT and mutant strains, or with *Bifidobacterium bifidum* as a control, for 18 h. The concentration of TNF-α cytokine was measured by ELISA.

TNF-α production by BMDCs was higher in *L. reuteri* 100-23 mutant strains which lack the SRRP and/or proteins involved in the glycosylation and secretion of the protein as compared to the *L. reuteri* 100-23 WT strain (**Figure 43**), with the *Asp2* mutant inducing the highest TNF-α production as compared to the WT strain. The *L. reuteri LSP* mutant yielded comparable levels of TNF-α production as the WT strain, suggesting that this protein is not involved in the induction of this inflammatory cytokine under the conditions tested. In addition, the *ftf* mutant induced increased TNF-α production compared to the WT strain further supporting the role of *L. reuteri* EPS in reducing inflammation.

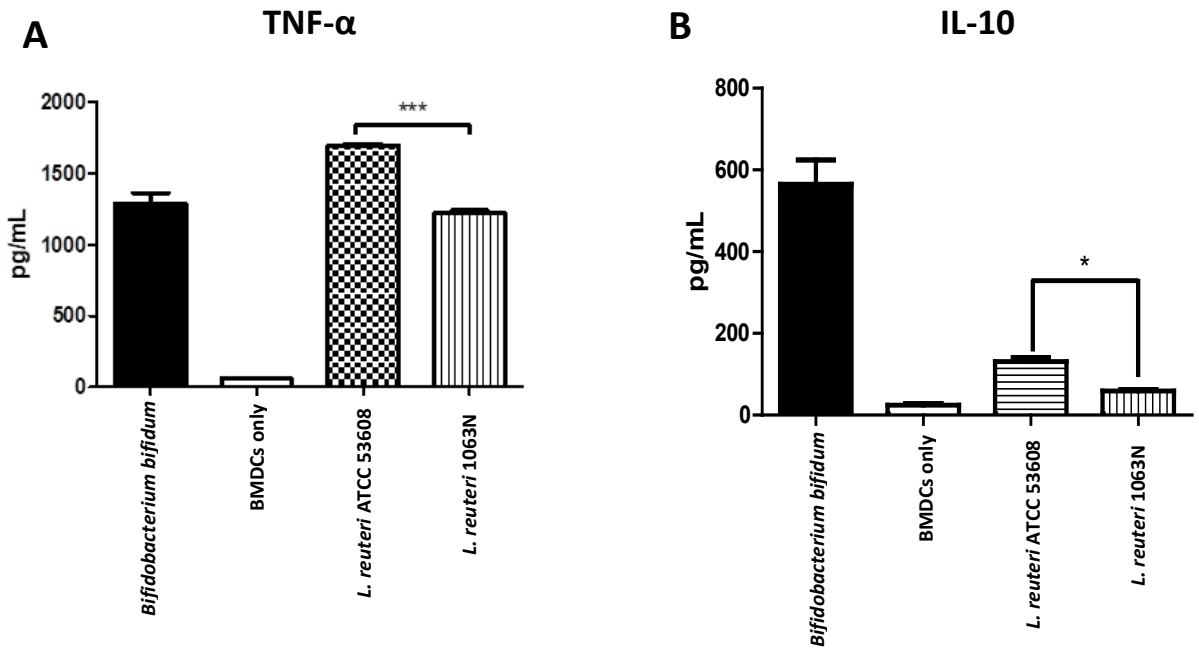

Figure 44: Effect of *L. reuteri* ATCC 53608 strains on cytokine production by BMDCs.

Mouse BMDCs were co-incubated with *L. reuteri* ATCC 53608 WT and mutant strain, or with *Bifidobacterium bifidum* as a control, for 18 h. The concentration of TNF-α cytokine was measured by ELISA.

We next investigated the effect of the pig isolate *L. reuteri* ATCC 53608 strain. The WT strain induced an increased production of TNF-α and IL-10 cytokine by BMDCs as compared to the MUB deficient mutant strain (**Figure 44A** and **Figure 44B**).

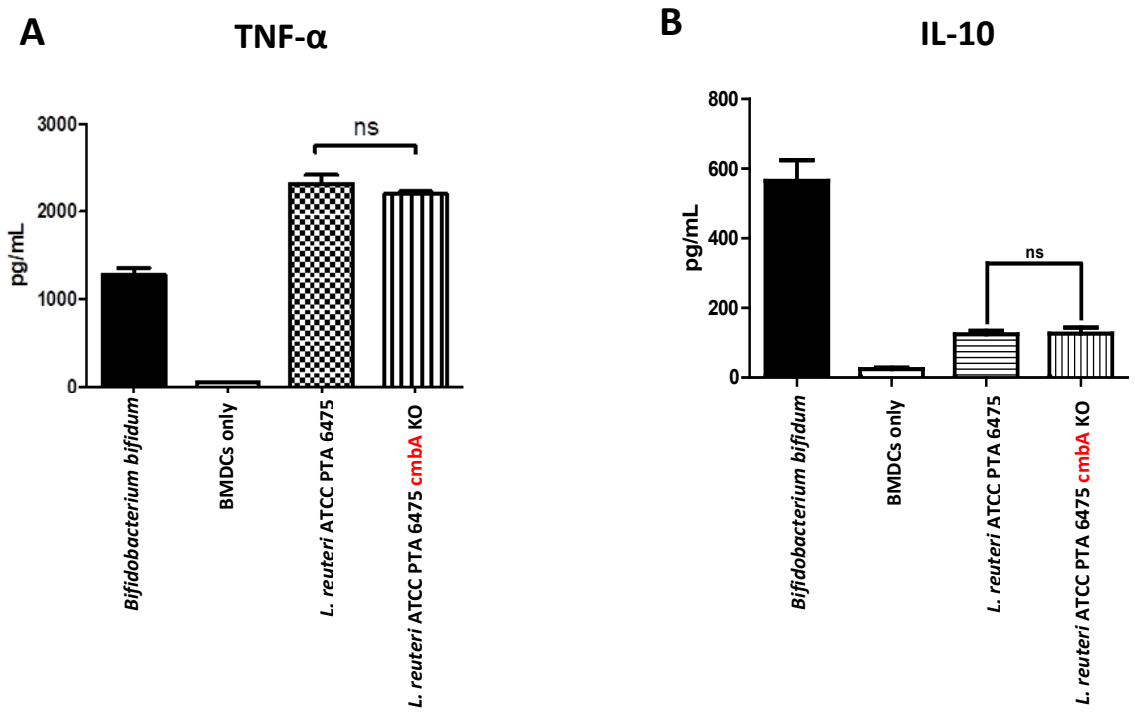

Figure 45: Effect of *L. reuteri* ATCC PTA 6475 strains on cytokine production by BMDCs.

BMDCs were co-incubated with *L. reuteri* ATCC PTA 6475 WT and mutant strain, or with *Bifidobacterium bifidum* as a control, for 18 h. The concentration of TNF-α cytokine was measured by ELISA.

Finally, the treatment of BMDCs with the cmbA *L. reuteri* ATCC PTA 6475 mutants led to TNF-α and IL-10 levels comparable to those obtained with the WT strain (**Figure 45A** and **Figure 45B**), suggesting that the cmbA protein does not contribute to immunomodulation of BMDCs in the conditions tested.

6.2.2 The role of L. reuteri strains in cytokine production by moDCs

The immunomodulatory properties of *L. reuteri* adhesins were also investigated using moDCs derived from human blood following treatment with *L. reuteri* 100-23 and ATCC 53608 WT and mutant strains. Pro-inflammatory (TNF-α, IL-1β, IL-8, IL-12) and anti-inflammatory cytokines (IL-10 and IL-4) concentrations were determined in supernatants of moDCs cultures by ELISA.

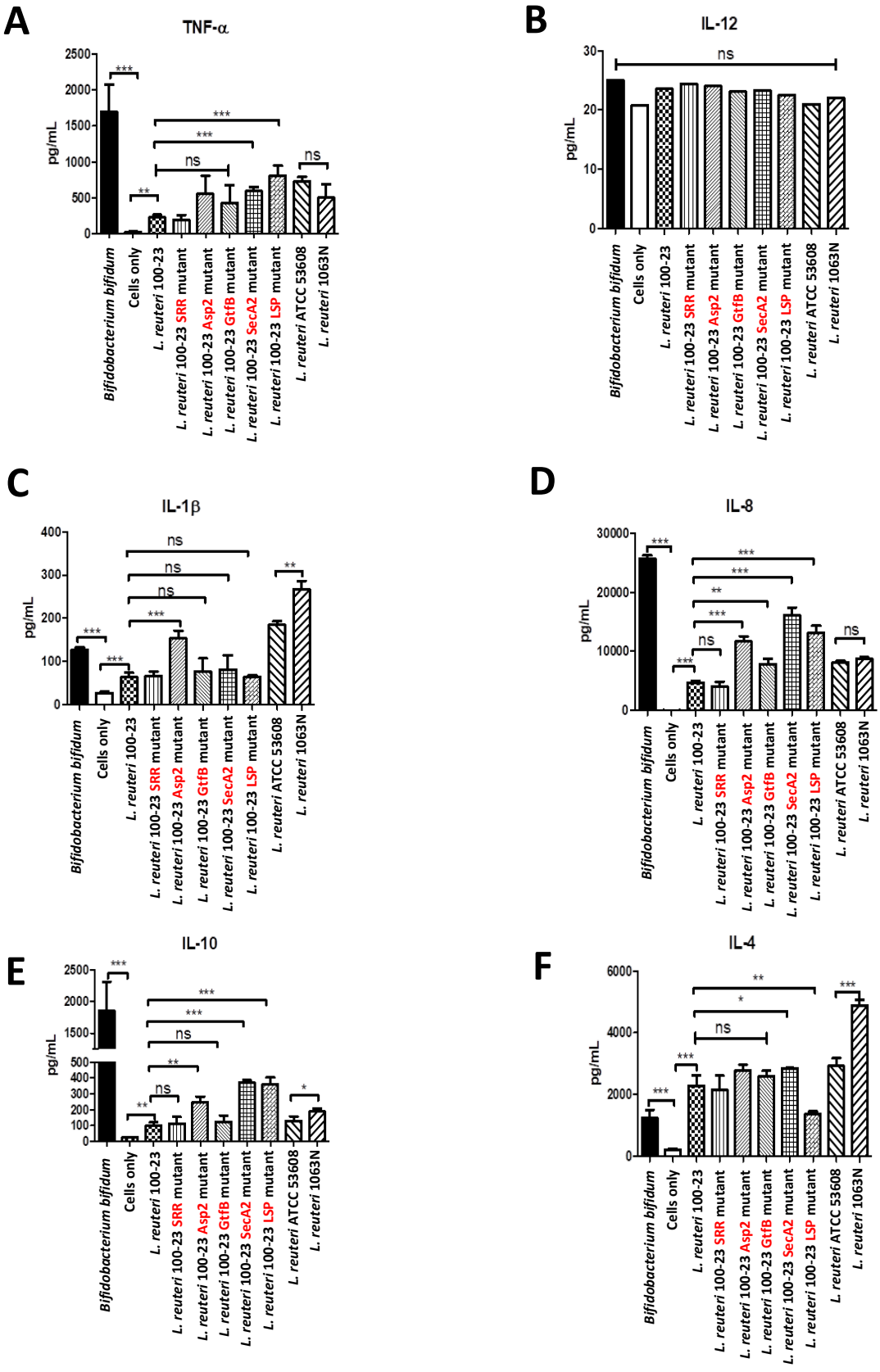
A
TNF-α
pg/mL
2500
2000
1500
1000
500
0
***
**
***
***
ns
ns
B
IL-12
pg/mL
30
20
10
0
ns
C
IL-1β
pg/mL
400
300
200
100
0
ns
ns
ns
***
***
**
D
IL-8
pg/mL
30000
20000
10000
0
***
***
**
***
ns
ns
E
IL-10
pg/mL
2500
2000
1500
500
400
300
200
100
0
***
***
ns
**
ns
**
*
F
IL-4
pg/mL
6000
4000
2000
0
**
*
ns
***
***
Bifidobacterium bifidum
Cells only
L. reuteri 100-23
L. reuteri 100-23 SRR mutant
L. reuteri 100-23 Asp2 mutant
L. reuteri 100-23 GtfB mutant
L. reuteri 100-23 SecA2 mutant
L. reuteri 100-23 LSP mutant
L. reuteri ATCC 53608
L. reuteri 1063N

Figure 46: Analysis of cytokine production following treatment of moDCs by *L. reuteri* 100-23 and ATCC 53608 strains.

Human moDCs were co-incubated with *L. reuteri* 100-23 and ATCC 53608 as well as their mutant strains, or with LPS as a control, for 24 h. The concentration of TNFα(A) IL-12 (B), IL-1β (C), IL-8 (D), IL-10 (E) and IL-4 (F) cytokines was measured by ELISA in 3 independent experiments. Mean values ± SD are shown. Statistical differences were analysed by the Student's t-test, with significance defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

L. reuteri strain 100-23 WT strain induced the production of IL-8 and TNF-α, IL-1β, IL-10 and IL-4, whereas production of IL-12 remained unchanged (**Figure 46**). No difference in TNF-α, IL-1β, IL-10 and IL-4 production was observed with the *SRR* and *Asp2* mutants, as compared to the WT strain (**Figure 46**), suggesting that SRRP may not be involved in the response of DCs to *L. reuteri* 100-23 strain under the conditions tested. The *GtfB* mutant, however, showed higher levels of IL-1β and IL-8 as compared to the WT strain. In addition, none of the *L. reuteri* strains tested modulated IL-12 cytokine secretion. Together these data show that the immunomodulatory properties of moDCs can be modulated by *L. reuteri* 100-23 cell surface adhesins.

L. reuteri ATCC 53608 strongly favoured the production of proinflammatory cytokines such as IL-4 and IL-1β when compared to the MUB-deficient 1063N mutant strain (**Figure 46**). This suggests that, *in vitro*, MUB contributes to an inflammatory rather than a stimulatory DC phenotype, in agreement with previously published data showing that MUB mediate pro-inflammatory effects by the induction of TNF-α and IL-1β cytokines[268]. Together these data indicate that *L. reuteri* strains share similar immunomodulatory properties on moDCs and BMDCs but with variable levels of induction.

6.2.3 The role of L. reuteri strains on maturation of BMDCs

To determine if *L. reuteri* cell surface proteins could play a role in DC maturation, we measured the effect of *L. reuteri* strains on the cell-surface expression of maturation-specific cell surface markers in BMDCs by flow cytometry.


100-23 WT
SRR mutant
GtfB mutant
Asp2 mutant
SecA2 mutant
LSP mutant
ftf mutant
ATCC PTA 6475 WT
cmbA mutant
ATCC 53608 WT
1063N
Unstimulated BMDCs
Isotype control
L. reuteri treated BMDCs
Cells %
CD40 Expression

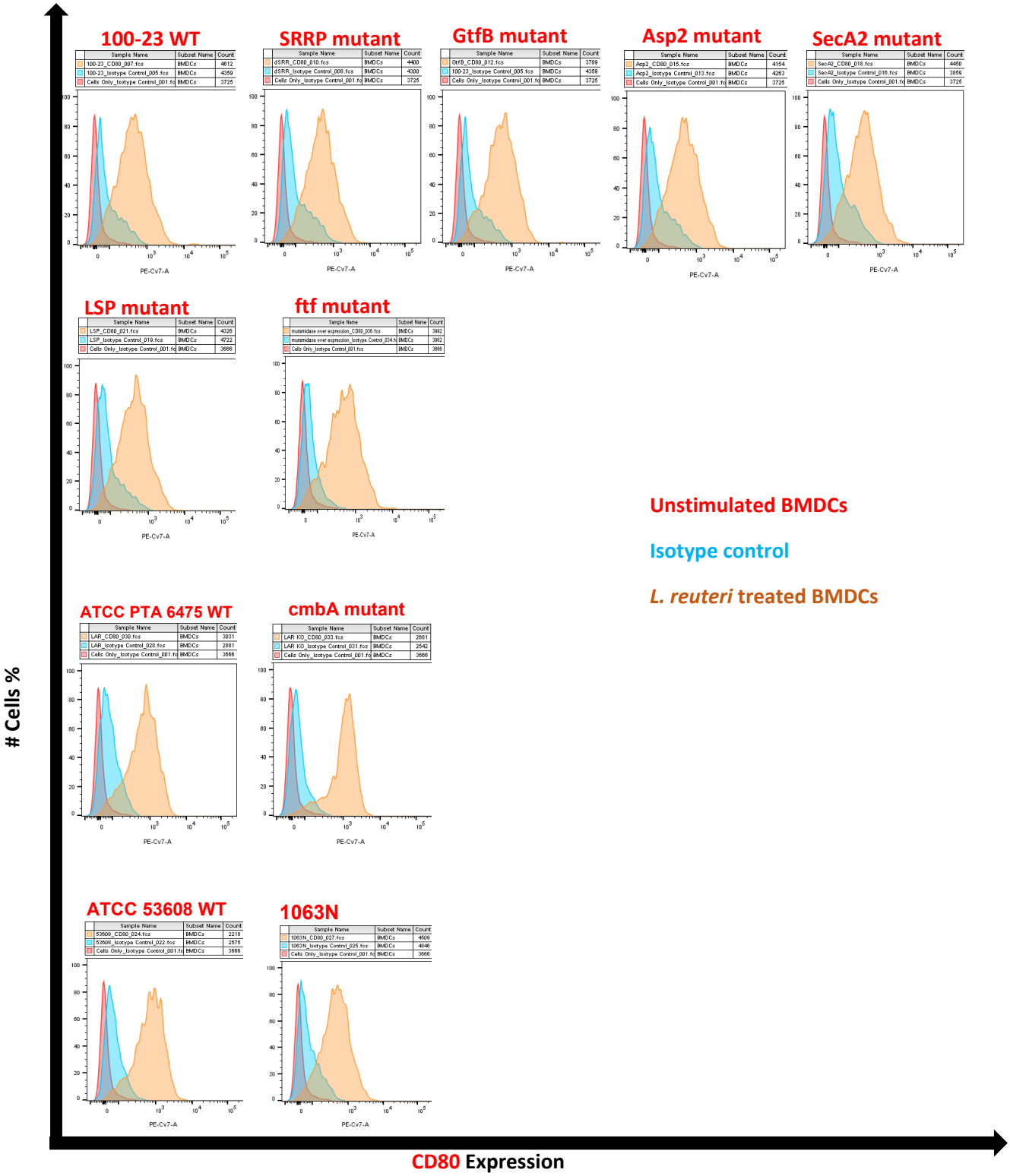

Figure 47: Effect of *L. reuteri* 100-23 strains on the surface expression of CD40 and CD80 in mouse BMDCs.

BMDCs were co-incubated with *L. reuteri* 100-23 for 18 h. Isotype control was used in the unstimulated cells (in blue).

The controls, unstimulated cells and isotype control showed no shift in fluorescence, confirming the specificity of the interaction with *L. reuteri* strains. BMDCs treated with all the *L. reuteri* strains upregulated the surface expression of the T-cell costimulatory molecules CD80 and CD40 (**Figure 47**). However, there was no difference in the expression of these activation markers between the different mutant strains of *L. reuteri* tested.

6.2.4 Internalisation of L. reuteri strains by BMDCs

We next investigated the role of cell surface components on the internalisation of *L. reuteri* strains by BMDCs. A control co-culture was performed at 4°C. At this temperature, energy dependent uptake as well as passive diffusion are blocked due to the rigidity of the membrane that does not enable passive internalisation to take place.

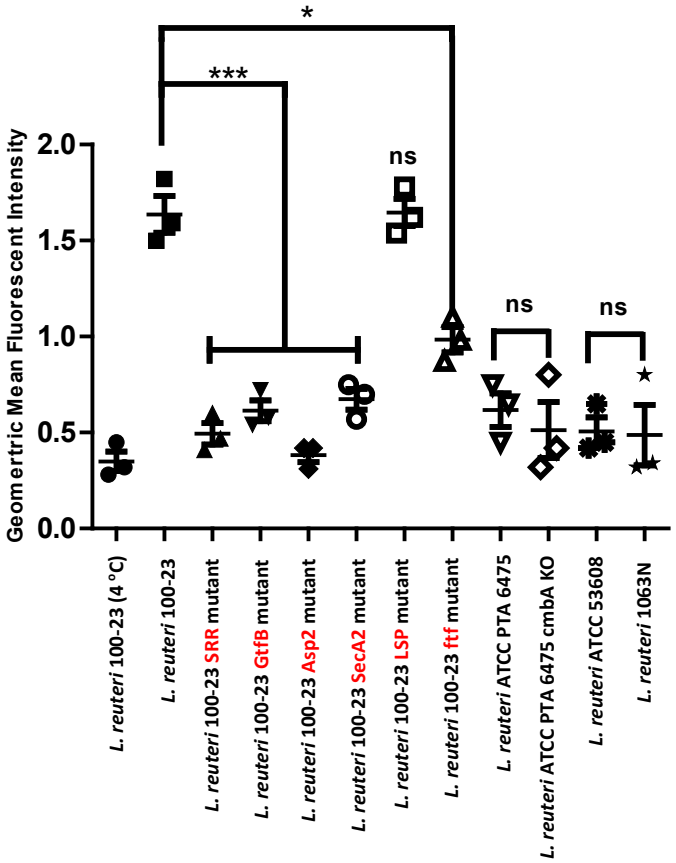

Figure 48: Analysis of *L. reuteri* strains uptake by BMDCs by imaging flow cytometry.

Bone marrow Isolated monocytes were differentiated in cell culture dishes at a density of 5.0×10^5 cells/mL in medium supplemented with GM-CSF for 7 days. BMDCs were then stained with CTV for 15 minutes at room temperature and stimulated with CFDA labelled *L. reuteri* strains. Coculture of CTV stained BMDCs and CFDA labelled bacteria was then performed at 37°C (unless stated otherwise) for 1 hour. Internalisation score calculated by Amnis IDEAS software: distribution of IS of at least 10,000 cells treated for 4 h at 37 °C or 4°C, and corresponding mean value of IS ± SD of three independent experiments.

Imaging flow cytometry analysis of host cell-bacteria interaction can be used to quantitatively address a variety of biological questions related to intracellular infection, including cell counting, internalisation score, and subcellular patterns of co-localisation. Here, we used Imaging flow cytometry to determine the internalisation score, defined as the ratio of fluorescence intensity of *L. reuteri* strains inside the BMDCs to the total fluorescence intensity of the BMDCs. *L. reuteri SRRP*, *aSec* mutants and the *ftf* mutant showed 3-fold and 2-fold lower internalisation level, respectively, as compared to *L. reuteri* 100-23 WT (**Figure 48**). No significant difference in the internalisation of bacteria by BMDCs was observed between the WT strain and the *LSP* mutant strain. In addition, as compared to the co-culture performed at 37°C, the co-culture carried out at 4°C showed a reduced internalisation score.

Furthermore, no significant difference in internalisation was observed between *L. reuteri* ATCC 53608 and 1063N strains. Similarly, ATCC PTA 6475 *cmbA* mutant was internalised at a same rate to the ATCC PTA-6475 WT strain (**Figure 48**). Finally, it is worth noting that the BMDCs were able to internalise *L. reuteri* 100-23 (from rodents) at higher rates compared to the ATCC 53608 (from pigs) and ATCC PTA 6475 (from humans) (**Figure 48**).

Taken together, these results suggest that, *in vitro*, the internalisation of *L. reuteri* 100-23 by BMDCs is dependent on the presence of the SRR protein and EPS on the surface of the bacteria.

6.3 Role of *L. reuteri*-derived bacterial extracellular vesicles (BEVs) on immunomodulation

Here we investigated the role of *L. reuteri*-derived BEVs on the immunomodulation of *L. reuteri* strains *in vitro*. BEVs were purified from *L. reuteri* 100-23 and ATCC 53608 grown at $OD6_{00nm}$ 0.6-1.2 by density gradient ultracentrifugation and characterised by proteomics, transmission electron microscopy (TEM) and by nanoparticle tracking analysis using Nanosight as described in **section 2.1.5.2**. TEM analysis showed that *L. reuteri* ATCC 53608 and 100-23-derived BEVs appeared rod-shaped (**Figure 49C** and **Figure 49D**).

Furthermore, the nanoparticle tracking analysis showed that the diameter of all *L. reuteri*-derived BEVs ranged from 30 to 250 nm (**Figure 49 A** and **Figure 49B**). The majority of *L. reuteri* ATCC 53608-derived BEVs showed a diameter of approximately 80-100 nm, while the majority of *L. reuteri* 100-23-derived BEVs showed a bigger size of approximately 100-130 nm.

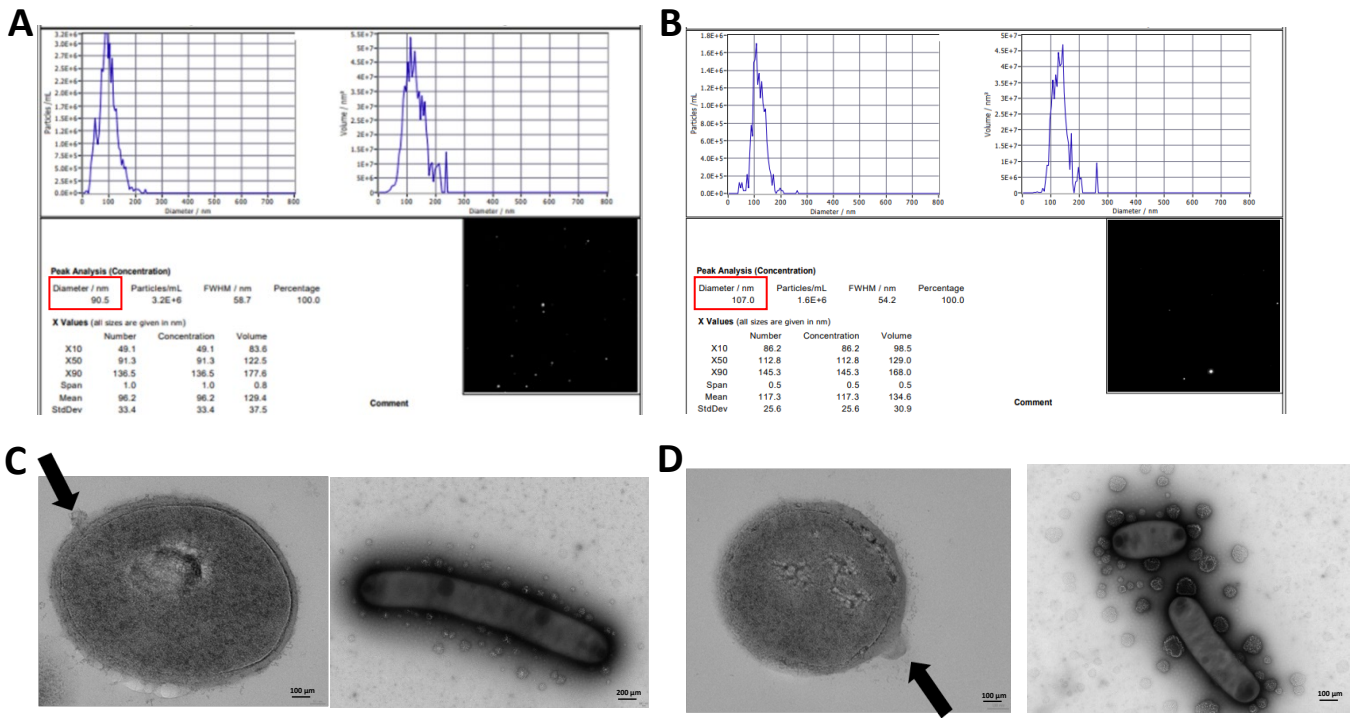

Figure 49: Characterisation of *L. reuteri*-derived BEVs.

L. reuteri ATCC 53608 **(A)** and *L. reuteri* 100-23 **(B)**-derived BEVs particle size was analysed by NanoSight. The main peak corresponds to the mode of the population. *L. reuteri* ATCC 53608 **(C)** and *L. reuteri* 100-23 **(D)**-derived BEVs were visualised by TEM. The arrows point towards BEVs.

We next performed proteomic analysis to identify the protein profile of purified *L. reuteri*-derived BEVs. A total of 57 and 555 proteins were identified in BEVs isolated from the *L. reuteri* 100-23 and ATCC 53608 strains, respectively. The subcellular localisation of these identified proteins showed that half of the proteins originated from the cytoplasm and with the rest of the proteins belonging to membrane and secreted proteins (**Figure 50**).

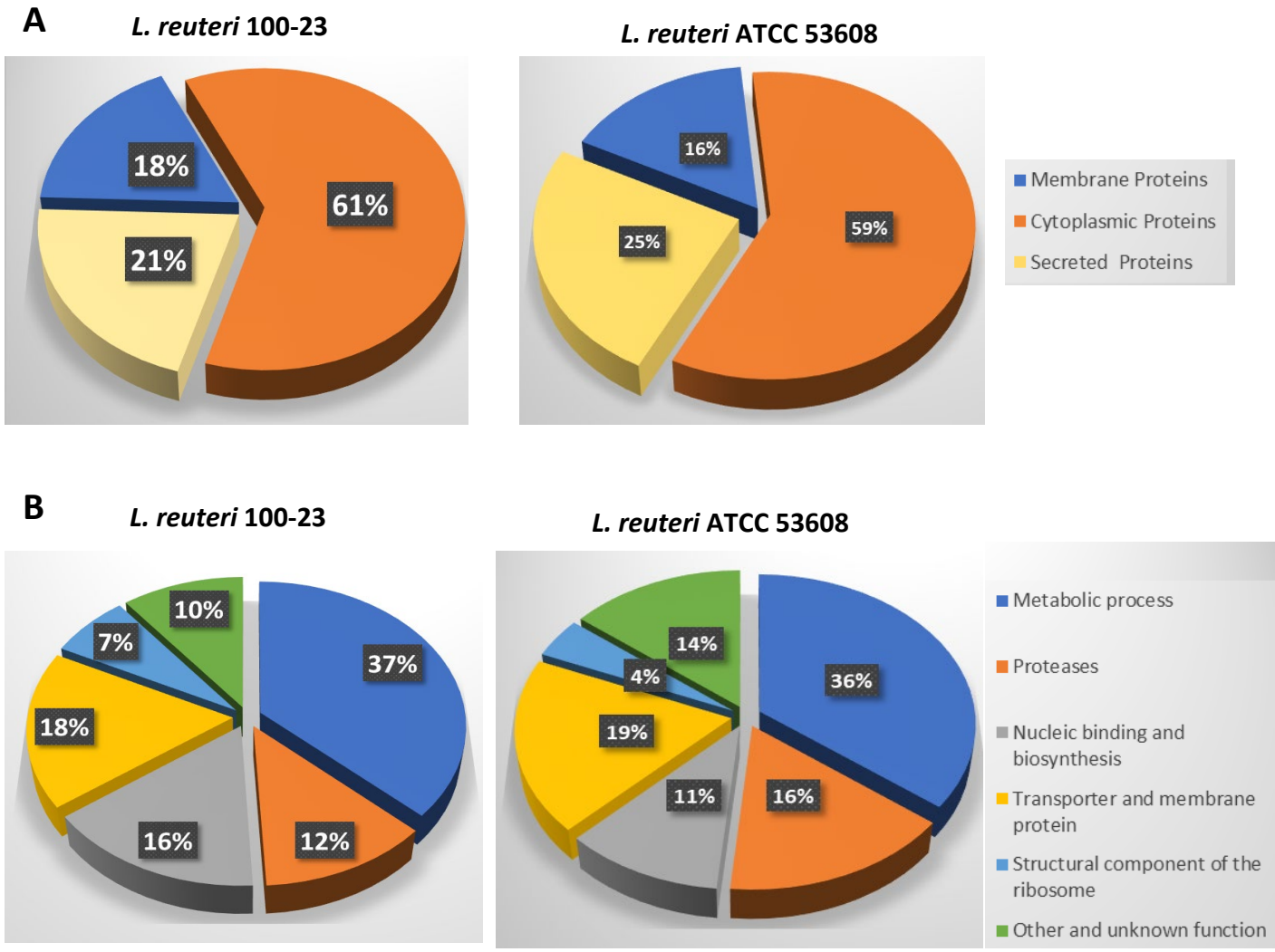

Figure 50: Proteomic analyses of *L. reuteri* BEVs.

(A) Subcellular localisation of the identified proteins present in the *L. reuteri* BEVs **(B)** Biological function classification of the identified proteins

According to the distribution of biological functions analysed by GO annotation, most of these proteins were classified into metabolic enzymes, proteases, nucleic-binding proteins, transporter and membrane proteins and structural components of the ribosome, suggesting that *L. reuteri* BEVs may be involved in metabolism, transporter activity, translation and transcription, signalling and stress, etc. Notably, several homologous proteins that were previously described as mediators of anti-inflammatory or beneficial effects in other probiotics or commensal bacterium were also observed in BEVs, such as glucosyltransferase, serine protease, 60 kDa chaperonin, elongation factor Tu and inositol polyphosphate phosphatase 1.

We also found MUB to be present in *L. reuteri* ATCC 53608-derived BEVs. However, SRRP was not identified in either of the strains tested in this study. Interestingly, proteins involved in the glycosylation and secretion such as GtfB, Asp1, Asp2, Asp3 and SecA were all identified in BEVs isolated from the ATCC 53608 strain. Altogether, these findings revealed that BEVs from *L. reuteri* strains carry immunoregulatory proteins, leading us to investigate the functions of these vesicles in the underlying mechanism of bacterium-host interactions.

We next tested the capacity of *L. reuteri* 100-23 and ATCC 53608 derived BEVs to modulate BMDC cytokine response in vitro. The number of BEVs were normalised to the size of the *L. reuteri* bacterial cell obtained using TEM (**Figure 49C** and **Figure 49D**). In this case, for every bacterial cell, we have treated the BMDCs with 10 BEVs.

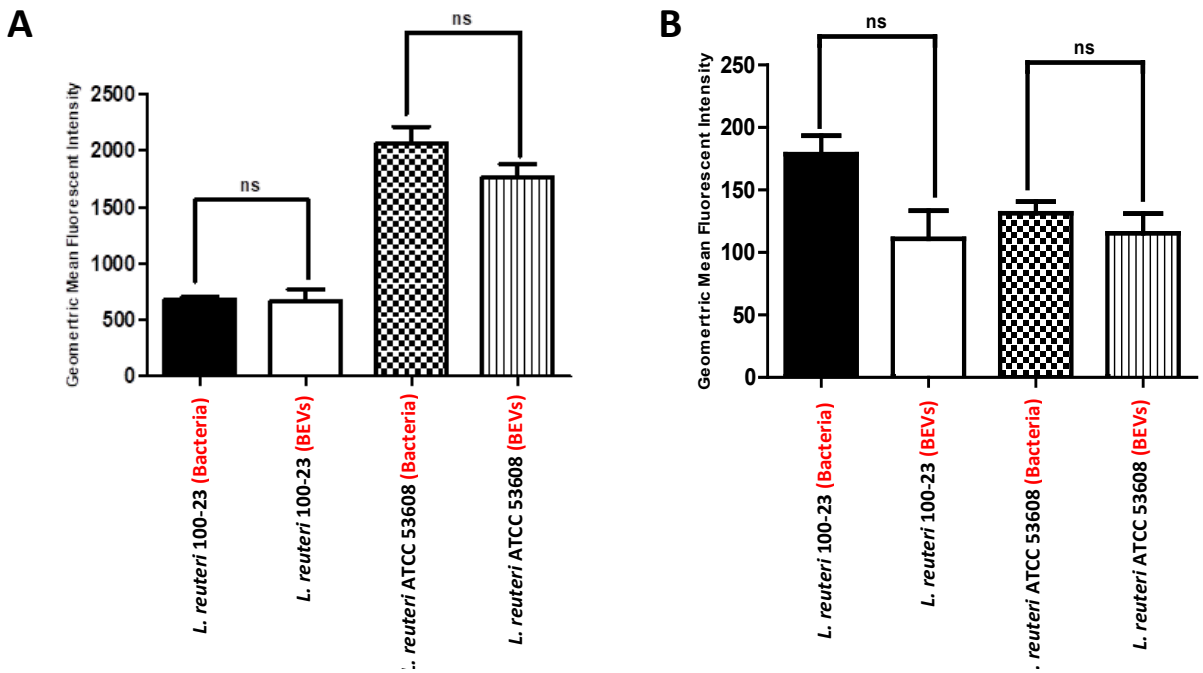

Figure 51: Effect of L. reuteri ATCC *L. reuteri*-derived BEVs on cytokine production by BMDCs.

Mouse BMDCs were co-incubated with *L. reuteri* ATCC 53608 or 100-23-derived BEVs or *L. reuteri* ATCC 53608 or 100-23 bacteria strains as controls, for 18 h. The concentration of TNF-α **(A)** and IL-10 **(B)** cytokines was measured by ELISA.

BEVs isolated from both *L. reuteri* ATCC 53608 and 100-23 strains were able to stimulate TNF-α (**Figure 51A)** and IL-10 (**Figure 51B)** production in BMDCs to levels comparable to that observed with the whole bacteria cells.

6.4 Effect of *L. reuteri* strains on THP-1 blue NF-κB production

To gain insights into the mechanisms involved in the induction of inflammation in DCs, THP1-NF-κB reporter cells were used to monitor and quantify the level of translocation of the transcription factor NF-κB from the cytosol to the nucleus. Heat inactivated *Listeria monocytogenes* (abbreviated here HKLM) was used as a positive control.

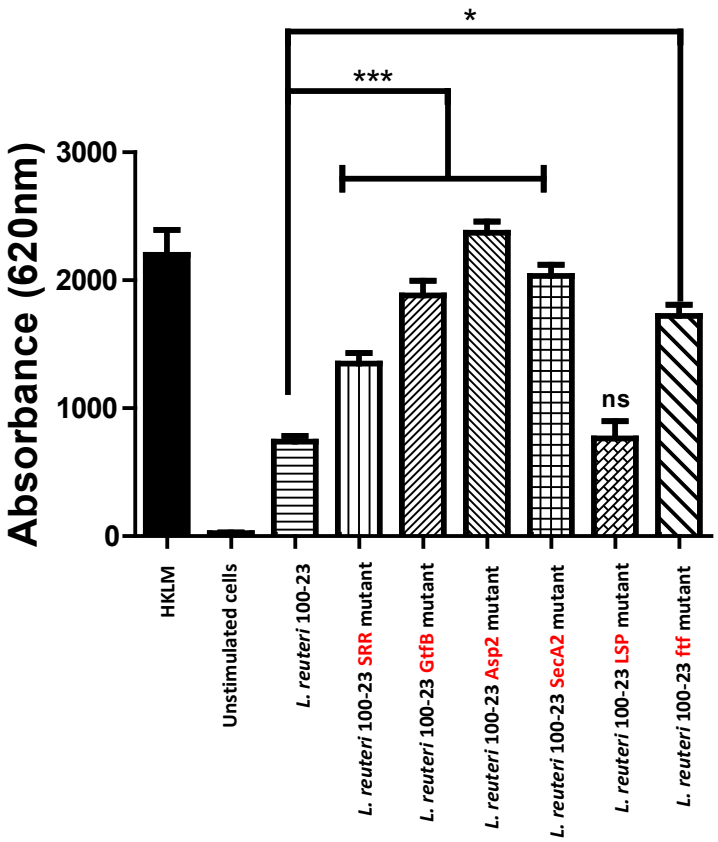

Figure 52: The role of *L. reuteri* 100-23 in the induction of NF-κB reporter cells.

THP1-Blue™ NF-kB cells, cells were incubated with *L. reuteri* strains or the positive control for the reporter assay, heat-killed *L. monocytogenes* (HKLM), at 37 °C in a 5% CO2 atmosphere for 8 h. 2 The production of was monitored from the medium with the QUANTI-Blue™ detection media. The experiments were carried out using technical replicates and at least 3 independent experiments.

Treatment of the reporter cells with *L. reuteri* 100-23 WT and mutant strains resulted in higher production of NF-κB as compared to the untreated cells (**Figure 52**). All *αSec* mutant strains were able to induce higher levels of NF-κB sa compared to the WT strain, implicating the SRR protein and its glycosylation and secretion system in the production of NF-κB by the reporter cells. Similarly, the EPS mutant was able to induce significantly higher levels of NF-κB as compared to the WT strain (**Figure 52**). However, there was no difference in NF-κB production between the *LSP* mutant and the WT strain.

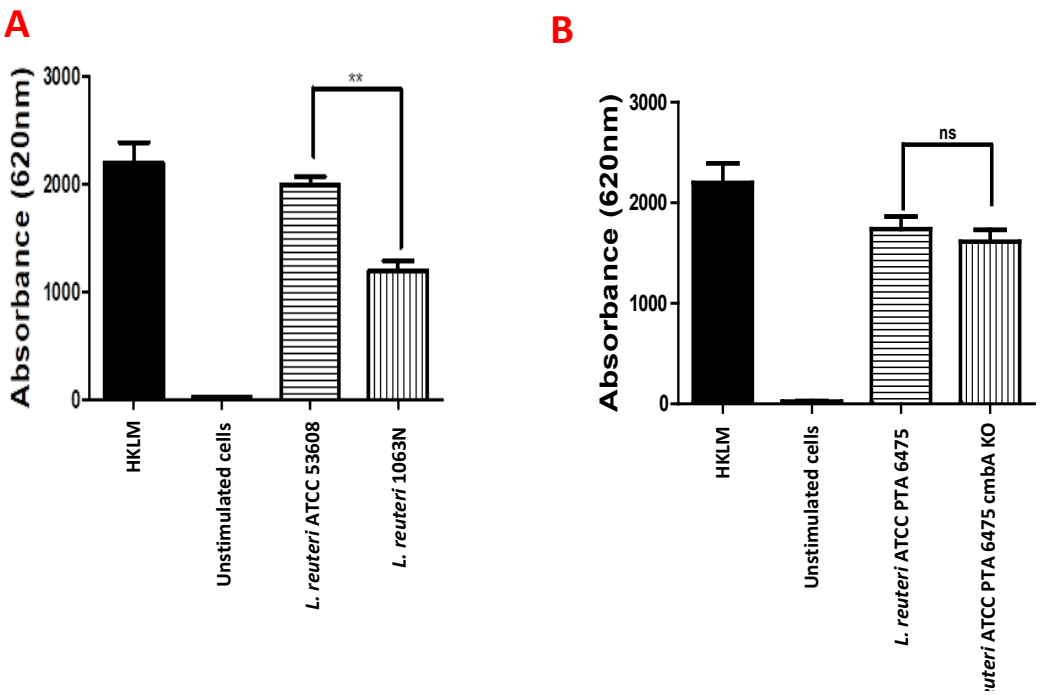

Figure 53: Effect of *L. reuteri* strains on NF-κB reporter cells.

THP1-Blue™ NF-kB cells, cells were incubated with *L. reuteri* ATCC 53608 **(A),** ATCC PTA 6475 **(B)** or the positive control for the reporter assay, heat-killed *L. monocytogenes* (HKLM), at 37 °C in a 5% CO2 atmosphere for 8 h. 2 The production of was monitored from the medium with the QUANTI-Blue™ detection media. The experiments were carried out using technical replicates and at least 3 independent experiments.

The ATCC 53608 WT strain was observed to induce higher levels of NF-κB as compared to the MUB deficient 1063N mutant strain whereas no difference in NF-κB induction was observed between the ATCC PTA 6475 WT and cmbA KO strains.

6.6 Discussion

Several studies have reported the probiotic effects of *L reuteri* strains on immune response of epithelium or immune cells *in vitro* or in preclinical models, although there is limited knowledge on how commensal *L. reuteri* regulates cytokine production and intestinal inflammation in mammals[575]. *L. reuteri* immunomodulatory effects are often strain-dependent, resulting in different DC activation profiles *in vitro*. For example, the production of TNF-α by LPS-activated monocytic cells has been reported to be downregulated by *L. reuteri* strains ATCC PTA 6475 and ATCC PTA 5289, whereas both *L. reuteri* strains ATCC 55730 and CF48–3A were shown to have an immuno-stimulatory effect *in vitro*[576, 577]. Spinler et. al. (2014) showed that *L. reuteri* strains from human-derived clades differed in respect to their ability to influence human cytokine production such as TNF-α, IL-1β, IL-7 and IL-12 by stimulated myeloid cells[578]. Here, we have shown that *L. reuteri* strains are capable of inducing both proinflammatory cytokines (such as TNF-α, IL-1β, IL-8 and IL-23) and pleiotropic anti-inflammatory cytokines (such as IL-10 and IL-4) by DCs harvested from humans and mice in a strain-specific manner. Some of the reported immunomodulatory effects are also attributable to the type of myeloid cells as well as the experimental conditions used in the *in vitro* assays, which is one of the limitations of *in vitro* work.

MoDCs originate from monocytes upon stimulation in the circulation and are the most abundant DC population during inflammation[579, 580]. Murine moDCs are believed to be functionally similar to the $CD14^{+}CD16^{-}$ and $CD14^{-}CD16^{+}$ human moDCs subpopulations *in vitro*[581]. However, although *in vitro*[582] and *in vivo*[583] evidence supports DC differentiation from monocytes, the possibility that monocytes represent precursors for DCs under physiological conditions remains a question of debate [584]. Indeed , it is widely accepted that *in vitro* monocyte differentiation systems cannot faithfully mimic the physiological conditions that control *in vivo* monocyte differentiation[584]. Nevertheless, moDCs represent an important tool for investigating the factors that control DC differentiation and might therefore provide information about the physiological conditions in which immune cell differentiation takes place[585-587]. On the other hand, *in vitro* granulocyte-macrophage colony-stimulating factor (GM-CSF) cultured DCs (or BMDCs) generates cells that share with DCs isolated from tissues the ability to present exogenous antigens to T cells and to respond to microbial stimuli by undergoing maturation and has

proven a useful model for molecular and clinical studies for the treatment of certain diseases[582]. Both moDCs and BMDCs have been shown to express similar surface markers such as alpha X (CD11c) and major histocompatibility complex II (MHCII) molecules[48,588], and respond to external stimuli in a similar manner[589-592]. However, it has been reported that BMDCs isolated from different hosts show different surface activation markers and cytokine production patterns[588]. Furthermore, mouse BMDCs are more efficient producers of the T cell-stimulating factor, IL-12 p70, compared to human BMDCs[588]. These apparent discrepancies may reflect the difference in cytokine production observed between murine BMDCs and human moDCs following *L. reuteri* strains treatment observed in this study.

Comparative genetic and genomic studies of *L. reuteri* 100-23 (from rodents) ATCC 53608 (from pigs) and ATCC PTA 6475 (from humans) showed fundamentally different trends of genome evolution in different hosts[31]. The unique genome content of *L. reuteri* lineages reflects the niche characteristics in the gut of their respective hosts. For example, rodent isolated strains have been shown to display elevated fitness in mice[31, 376], and biofilm formation in the forestomach is restricted to strains from rodent lineages[378]. Here, we showed that the DC activating potential of *L. reuteri* 100-23, ATCC PTA 6475 and ATCC 53608 strains by the co-stimulatory molecules CD40 and CD80. Successful T cell activation occurs when their T-cell receptors (TCRs) are engaged and triggered by ligands on the surface of antigen-presenting cells such as DCs[593]. In addition to T cell receptor engagement, costimulatory signals are essential for T cell survival, expansion and acquisition of effector functions[593]. In the future, it will be of interest to assess the immunomodulatory effect of these strains on LPS pre-treated cells, to recapitulate the inflammation status which is often associated with an impaired gut barrier and a translocation of bacteria.

We showed that $SRRP_{100\text{-}23}$ and MUB_{53608} contribute to the immunomodulatory effects of *L. reuteri* 100-23 and ATCC 53608 *in vitro* by influencing the secretion of pro-inflammatory cytokines such as TNF-α as well as the T-lymphocyte polarising cytokine IL-23, suggesting that these adhesins may play a role in gut homeostasis. Indeed, to fend off bacterial infections and inflammation, activated DCs must respond rapidly to external stimuli and changes in their microenvironment[594]. Most types of signals induce cellular responses by binding to specific cell-surface receptors such as TLRs or C-type-lectins that respond to occupancy by triggering one or more signal transduction pathways[595]. As shown in **section X**, C-type lectin receptors such as SIGNR1 and Dectin-2 may be involved in the interaction with *L. reuteri* EPS and glycosylated adhesins.

One of the most common responses to receptor engagement is the activation of transcription factors and the synthesis of new proteins. Among the transcription factors, the active heterodimer p50/p65 form of nuclear factor NF-κB plays a central role in immunological processes by inducing expression of genes involved in inflammatory responses[596]. For example, mature DCs have been shown to express high levels of the NF-κB family of transcription factors[597] and signalling by members of the TNF-α receptor family, such as CD40, results in activation of NF-κB[598, 599]. Here, we demonstrated the role of the *L. reuteri* adhesins in the induction NF-κB signalling pathway. Both $SRRP_{100\text{-}23}$ and MUB_{53608}, but not cmbA, were shown to be important in the induction of this induction pathway

Together these results indicated that *L. reuteri* glycosylated $SRRP_{100\text{-}23}$ and MUB_{53608} contribute to the development of different NF-κB-dependent immune responses by DCs.

In addition to a potential direct interaction with immune cells, the complex microbiota-host communication can be mediated through active mediators secreted by the gut microbiota, such as SCFAs, histamine and indole[600] or EPS[387]. For example, TNF-α production in mice[601] and by human PBMCs[602] was reduced by soluble factors derived from *L. reuteri* CRL1098. Moreover, the production of IL-6, IL-10, and IL-23 induced by LPS was downregulated while the secretion of regulatory cytokine TGF-β remained unaffected by human moDCs differentiated in the presence of *L. reuteri* DSM 17938 soluble factors[603]. Here, we have shown the importance of *L. reuteri* 100-23 EPS in NF-κB induction, enhanced CD40 and CD80 expression, and secretion of anti-inflammatory cytokines IL-10. This anti-inflammatory role of *L. reuteri* 100-23 EPS supported by previous work showed colonisation of the mouse gut by the strain lacking EPS resulted in decreased proportions of regulatory T cells (Foxp3+) in the spleen that would otherwise generate immunological tolerance towards the commensal[387].

Using imaging flow cytometry, we demonstrated the capacity of BMDCs to internalise *L. reuteri* strains and the role of cell-surface adhesins and EPS in this process. Imaging flow cytometry has been applied to address questions used in infection biology, in particular, to study mechanisms underpinning infections induced by intracellular pathogens[604]. This methodology makes it possible to analyse hundreds of quantified features for hundreds of thousands of individual cellular or subcellular events in a single experiment[605, 606]. We showed that the BMDCs were able to internalise *L. reuteri* 100-23 (from rodents) at higher rates compared to the ATCC 53608 (from pigs) and ATCC PTA 6475 (from humans), which may reflect some degree of host-specificity.

Our results also demonstrate that the cell-surface expressed adhesin $SRRP_{100-23}$ as well as EPS have the potential to promote the internalisation process of *L. reuteri* 100-23 by BMDCs (isolated from mice). DCs constitute specialised regions of the intestine and are known to continuously sample the intestinal luminal content[607, 608]. To present antigens that activate $CD4^+$ T helper cells, DCs internalise and process microorganisms[609, 610]. During the process of internalisation, microorganisms are encapsulated by the plasma membrane forming an intracellular vacuole known as the phagosome[611]. The phagosome undergoes a series of regulated fusion events with endocytic organelles, first with endosomes and subsequently with lysosomes, modifying their composition. During this process, internalised material is degraded into small fragments and loaded onto major histocompatibility complex (MHC class II)[611]. The type and quality of T-cell responses are additionally determined by costimulatory signals such as CD40, CD80 and CD86[598, 612].

Together these data suggest that $SRRP_{100-23}$ or EPS may mediate NF-κB-dependent cytokine production through internalisation of *L. reuteri* 100-23 by DCs. However, more work is warranted to confirm the biological role of SRRP in the internalisation of *L. reuteri* 100-23 by DCs in mice.

In a healthy state, the host's immune response to the gut microbes is strictly compartmentalised to the mucosal surface[613]. The mucus layer organised around the hyperglycosylated mucin MUC2 not only offers protection by static shielding, but also constrains the immunogenicity of intestinal antigens by imprinting enteric DCs towards an anti-inflammatory state[614]. Among various bacteria-derived mediators, BEVs have been shown to play an important role in intercellular crosstalk or signalling[408]. Here, we first showed that both the pig and rodent isolates can produce BEVs whose concentration and size are consistent with the typical results characterised by NTA analysis of *L. reuteri*-derived BEVs[409]. Here, we showed that BEVs purified from *L. reuteri* strains isolated from pig (ATCC 53608) and rodent (100-23) can induce both inflammatory and anti-inflammatory cytokine production by DCs *in vitro* as also shown using *L. reuteri* whole strains. Consistent with our data, BEVs derived from several probiotics and commensal bacteria, such as *Bacteroides fragilis*[615], *Akkermansia muciniphila*[616], *Escherichia coli* Nissle 1917[617], *Bifidobacterium longum*[618] and *L. paracasei*[619], have been shown to exert similar pro-inflammatory and anti-inflammatory effects as their parental bacteria, supporting a role of BEVs in their communication with immune cells. Due to the unique nano-scale structure of the lipid membrane-encapsulated vesicles, BEVs can drive the long-distance transport of interior molecules throughout the intracellular compartments in a concentrated, protected and targeted manner[620]. BEVs are known to contain various bioactive molecules of the parental bacteria, including proteins and nucleic acids[409].

Membrane vesicles from Gram-negative bacteria have also been shown to induce a range of cellular responses via lipopolysaccharide (LPS) lipopolysaccharides and EPSs[621, 622]. LPS is the hallmark of the outer membrane vesicles (OMVs) and was one of the first components observed in association with MVs in Gram-negative bacteria[623]. For example, *Escherichia coli* (*E. coli*) OMVs contain LPS that can drive TLR4-dependent CXCL8 production in human epithelial cells[624]. In addition, enterohemorrhagic *E. coli* OMVs allow efficient delivery of LPS into the host cell cytoplasm, resulting in inflammatory responses and cell death[625]. However less is known about the presence of cell wall polysaccharides derived from Gram-positive bacteria and their role in mediating immune response.

Electron microscopy studies have been used to show that part of the protein content detected in the EPS from *Phyllophora antarctica* NF_3 was released to the media through membrane vesicles[626]. *Bacteroides fragilis* has also been shown to release capsular polysaccharide and EPS via its OMVs and induce immunomodulatory effects and prevent experimental colitis in mice[615]. It is therefore possible that *L. reuteri* BEVs modulate the immune system through surface adhesins and possibly EPS.

Proteomic analysis was further performed to determine the protein profile of *L. reuteri* derived BEVs. Here we showed that most of the proteins present in *L. reuteri* BEVs are cytoplasmic and the rest belonged to the membrane and secreted proteins, which are similar to those observed in the proteome of BEVs isolated from other *L. reuteri* strains[409]. The functions of all the BEVs proteins identified in this study were categorized according to the GO annotation based on the biological functional hierarchy[627]. Most of the cytoplasmic proteins found were ribosomal or metabolic proteins, which are similar to those observed in the proteome of Gram-positive, Gram-negative bacterial OMVs and mammalian microvesicles[409, 628-630]. Although we cannot completely exclude the possibility of contamination derived from the small proportion of lysed bacterial cells within the culture, several cytoplasmic proteins, including pyruvate dehydrogenase, were described to be synthesized by the membrane-bound ribosomes complex, which is presumed to be the protein-secretion machinery[631]. In addition, it has been suggested that many of the plasma-membrane enzymes and electron-transport components (e.g. ATPase and dehydrogenase) can be excreted from Gram-positive bacteria[632]. Taken together with previous studies on Gram-negative bacterial OMVs, this observation suggests that *L. reuteri*-derived BEVs might facilitate the transfer of proteins involved in translation and metabolic enzymes to their own or other bacteria[628, 633].

The discovery of the MUB protein in the vesicles isolated from the ATCC suggest that BEVs may also play a role in the interaction with GI barrier (mucus and epithelial cells) and immune cells. Indeed, we have

shown that MUB increases the capacity of *L. reuteri* ATCC 53608 strains to interact to different tissues of the GI tract (**Chapter 4**). The ability of *L. reuteri* ATCC 53608 strain to interact with intestinal lectins *in vitro* was shown to be dependent on the surface expression of MUB (**Chapter 5**). In addition, in this chapter, we also highlighted the role of MUB in the immunomodulatory properties of the ATCC 53608 *in vitro* by influencing the secretion of pro-inflammatory cytokines such as TNF-α and IL-20.

Interestingly, despite the presence of proteins involved in the glycosylation and secretion of the SRRP protein, SRRP was not detected in the BEVs. In proteomic analysis, the score assigned to each protein, and therefore the probability for the match to be right, depends on a number of parameters, such as spectral quality and mass accuracy[634]. In addition, the more peptides are assigned to a given protein, the higher the protein score will be[635]. In our preliminary proteomics analysis (data not shown), SRRP is the fifth protein to be identified by the Mascot database even from In-gel digestion of proteins which suggests low numbers of peptide sequences identified because in the Mascot database, all peptides are analysed simultaneously and only the most abundant/ionisable peptides are observed (www.matrixscience.com). SRRP is the primary cell wall-associated protein of strain 100-23[378] and ATCC 53608[394] that is secreted through an accessory SecA2–SecY2 pathway. Given the fact that we detected components of the accessory secretion system, therefore, it is possible that the reason we did not detect SRRP in our proteomic analysis of *L. reuteri*-derived BEVs might be because amount of SRRP in the BEVs was insufficient or because it was masked by another protein/s.

Taken together, these data provide new insights into the mechanisms by which *L. reuteri* strains exert immunomodulatory properties via the interaction of *L. reuteri* host- specific adhesins and EPS with DCs. The BEVs or glycosylated adhesins expressed on the cell surface of *L. reuteri* strains may contribute to the maintenance of the symbiotic relationship with the host by acting as a natural adjuvant, thus provoking antigen-specific adaptive immune responses by DC through the development of effector and memory T-lymphocytes with sufficient stimulatory potential.

CHAPTER 7: ROLE OF C-TYPE LECTINS IN THE INTERACTION BETWEEN *L. REUTERI* STRAINS AND IMMUNE CELLS

7.1 Introduction

As described in **Chapter 6**, *L. reuteri* strains showed strain-specific immunomodulatory properties on DCs *in vitro* involving glycosylated cell-surface components. Mounting of an effective and durable adaptive response against microbes requires activation of antigen-presenting cells (APCs)[636]. APCs such as DCs, macrophages and B cells are found throughout the human body including tissues, barrier sites and in the circulation[637]. These cells are important for processing external signals to instruct both local and systemic responses toward immune tolerance or immune defence[638]. APCs express an extensive repertoire of pattern-recognition receptors (PRRs) to detect and transduce these signals[639, 640]. C-type lectins (CTLs) comprise a subset of PRRs dedicated to recognising an array of glycans, including those expressed by commensal and pathogenic bacteria (**see section 1.4.1**).

Interactions of CTLs with specific glycans occur through one or more carbohydrate recognition domains (CRDs) in a Ca^{2+}-dependent manner[641]. CTLs expressed on APCs can be categorised based on conserved amino acid motifs in their CRDs that determine their glycan specificity and Ca^{2+} coordination. CTLs with an EPN (Glu-Pro-Asn) amino acid motif in their CRD, such as Dectin-2, DC-SIGN, langerin and mannose receptor preferentially bind glycans with equatorial 3- and 4-hydroxyl groups such as mannose, fucose, and GlcNAc residues. On the other hand, CTLs such as human Mincle (a macrophage inducible Ca^{2+}-dependent CTL) or rat mannose-binding lectin A, with a QPD (Gln-Pro-Asp) motif preferentially bind glycans with axial 4-hydroxyl groups such as galactose and GalNAc terminated glycans[642, 643]. In addition, some CTLs such as the mouse Dectin-1, contain no EPN or QPD sequence in the CRD and do not require Ca^{2+} for the interaction[644]. Therefore, it has been suggested that Dectin-1 has a recognition mode that is distinct from that of other C-type lectins.

Many CTL-dependent bacterial recognition events promote bacterial clearance, whereas other interactions are exploited by bacteria to enhance their pathogenic potential[645]. CTLs are also densely concentrated at APC dendrites that sample the environment across intact barrier sites. This suggests an–as yet–underappreciated role for CTL-mediated recognition of microbiota-expressed glycans in maintaining tolerance at barrier sites. To determine the role of *L. reuteri* glycosylated cell-surface adhesins in the ability of the strains to interact with CTLs and induce an immune response, we tested a range of WT and mutant *L. reuteri* 100-23 strains on CTLs. These include *L. reuteri* 100-23 *aSec* mutants, *SecA2*, *Asp2*, *GtfB* as well as insertion mutants targeting cell surface proteins SRRP a large surface

protein (*LSP*) mutant. In addition, based on results from Chapter 6 implicating *L. reuteri* 100-23 EPS in the interaction with mice forestomach, and the potential for EPS to contribute to the interaction of *L. reuteri* strains with C-type lectins, via a direct interaction or by masking glycosylated adhesins, we included the *ftf* mutant lacking EPS in this work.

7.2 Interaction of *L. reuteri* 100-23 strains with C-type lectins (CTLs)

To test whether CTLs may be implicated in the interaction of *L. reuteri* 100-23 glycosylated cell-surface adhesins with DCs (as shown in **Chapter 6**), the binding of *L. reuteri* WT and mutant strains was tested to reporter cells expressing individual mouse CTLs, mDectin-1, mDectin-2 and SIGNR1 (murine homolog of human DC-SIGN), on their cell surface.

Following ligand recognition of CTLs, CD3ζ-mediated activation of nuclear factor of activated T-cells (NFAT) and induction of the IL-2 promotor/lacZ, and β-galactosidase activity was measured in a colorimetric assay in the presence of CPRG, a substrate for β-galactosidase**.** Scleroglucan, Furfurman and Hafnia alvei LPS (*H. alvei*) were used as positive controls for the mDectin-1, mDectin-2 and SIGNR1 reporter cells, respectively. Furfurman, cell wall preparation extracted from *Malassezia furfur,* binds to CTL receptors, in particular Dectin-2[646], and *H. alvei* LPS is an agonist of SIGNR1[431]. Scleroglucan, a high molecular weight polysaccharide, is a specific ligand for Dectin-1[647]. In addition, to confirm the protein-glycan specificity of the interaction, we used reporter cells expressing carbohydrate-binding defective mutant of SIGNR1 and Dectin-2, generated by two missense mutations in the carbohydrate recognition domains leading to amino acid substitutions QPD (for SIGNR1) and QPD (for Dectin-2) [648]. Mock cells that do not express CTLs on their surfaces were used as a negative control.

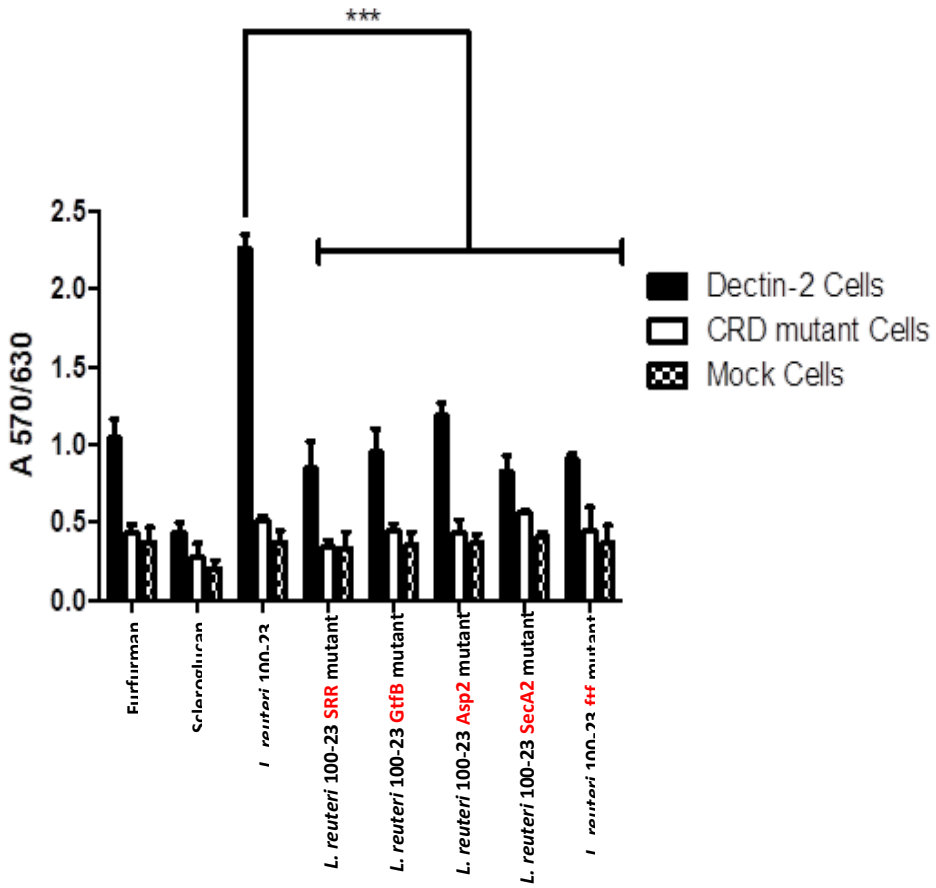

Figure 54: Interaction of *L. reuteri* 100-23 wild-type and mutant strains with mDectin-2 reporter cells.

Bacteria strains were adsorbed onto 96-well microplates. Scleroglucan (10 µg/mL) and furfurman (10 µg/mL) were used as negative and positive control, respectively. β-galactosidase activity of BWZ.36 cells (expressing Dectin-2, CRD mutant or mock cells) was determined using CPRG as substrate. Absorbance was detected (A570/630 nm) with a Bio-Rad Benchmark Plus microtiter plate reader. Mean values ± SD are shown. Statistical differences were analysed by the Student's t-test, with significance defined as ∗p < 0.05, ∗∗p< 0.01, ∗∗∗p< 0.001, and ∗∗∗∗p< 0.0001.

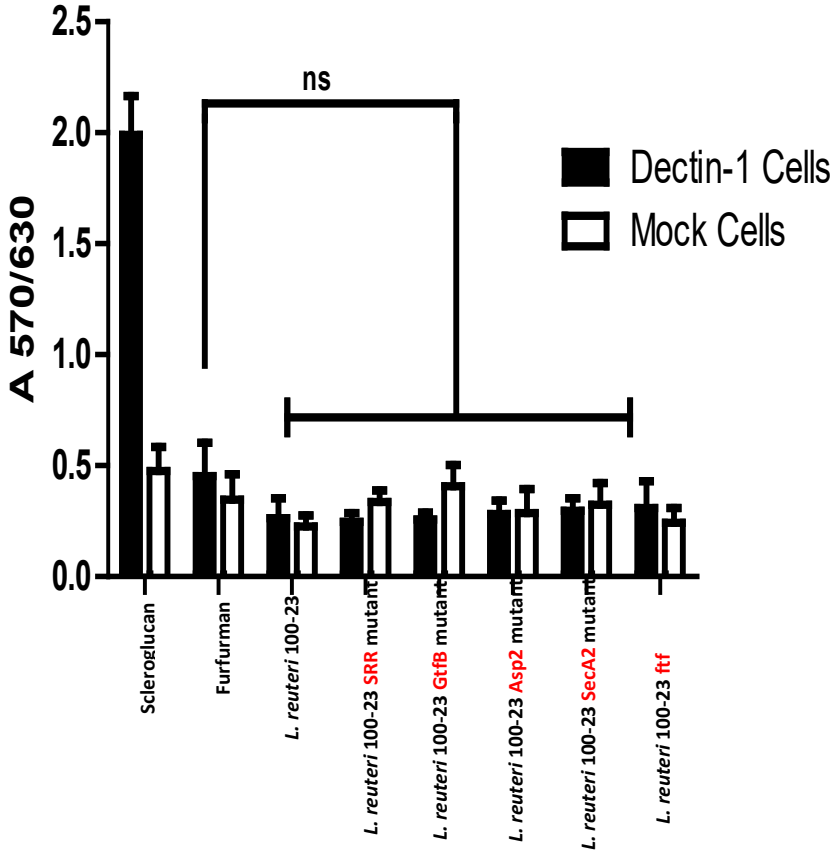

Figure 55: Interaction of *L. reuteri* 100-23 wild-type and mutant strains with mDectin-1 reporter cells.

Bacteria strains were adsorbed onto 96-well microplates. Scleroglucan (10 µg/mL) and furfurman were used as positive and negative control, respectively. β-galactosidase activity of BWZ.36 cells (expressing Dectin-1 or mock cells) was determined using CPRG as substrate. Absorbance was detected (A570/630 nm) with a Bio-Rad Benchmark Plus microtiter plate reader. Mean values ± SD are shown. Statistical differences were analysed by the Student's t-test, with significance defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p< 0.0001$.

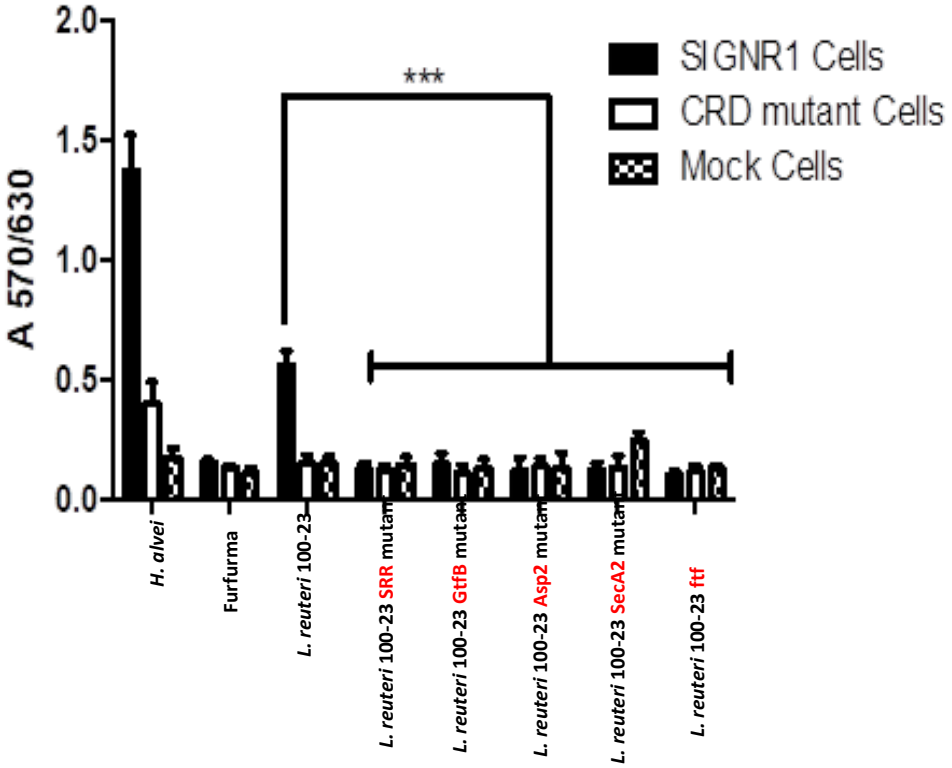

Figure 56: Interaction of *L. reuteri* 100-23 wild-type and mutant strains with SIGN-R1 reporter cells.

Bacteria strains were adsorbed onto 96-well microplates overnight at 4°C. *H. alvei* LPS (10 µg/mL) and furfurman (10 µg/mL) were used as positive and negative control, respectively. β-galactosidase activity of BWZ.36 cells (expressing SIGN-R1, QPD mutant or mock cells) was determined using CPRG as substrate. **(A)** wild-type **(B)** *SRR* mutant **(C)** *GtfB* mutant **(D**) *Asp2* mutant **(E)** *SecA2* mutant and **(F)** summary. Absorbance was detected (A570/630 nm) with a Bio-Rad Benchmark Plus microtiter plate reader. Mean values ± SD are shown. Statistical differences were analysed by Student's t-test, with significance defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

No binding was observed between *L. reuteri* 100-23 (WT and mutant strains) and Dectin-1 reporter cells, whereas the positive control, scleroglucan, showed higher binding affinity towards the reporter cells as indicated by the level of β-galactosidase activity (**Figure 55**). In contrast, *L. reuteri* 100-23 WT strain significantly bound to Dectin-2 (**Figure 54**) and SIGN-R1 reporter cells as compared to the mock reporter cells ($p < 0.0001$) (used as a negative control) (**Figure 56**). When *L. reuteri* 100-23 WT strain was tested on reporter cells expressing Dectin-2 or SIGN-R1 QPD mutants, in which the glycan-binding activity had been abolished, the binding was significantly reduced, indicating the specificity of the protein-glycan interaction (**Figure 54** and **Figure 56**).

All *L. reuteri* 100-23 mutant strains tested showed reduced binding to Dectin-2 and SIGN-RI reporter cells as compared to the WT strain (**Figure 54 and Figure 56**). In particular, the *L. reuteri* 100-23 WT strain induced 36% ($p< 0.001$) and 63% ($p < 0.001$) more production of β-galactosidase than did the *L. reuteri* 100-23 SRRP mutant in the Dectin-2 and SIGN-RI reporter cells, respectively (**Figure 54 and Figure 56**). These results suggest that $SRRP_{100\text{-}23}$ plays an important role in binding to cell surface receptors found on immune cells such as DCs.

To further determine the role of Dectin-2 in the mechanisms mediating the interaction of *L. reuteri* surface adhesins with BMDCs, flow cytometry was used to study the direct interaction of recombinant human Dectin-2 produced in our Lab (hDectin-2) to *L. reuteri* 100-23 WT and mutant strains.

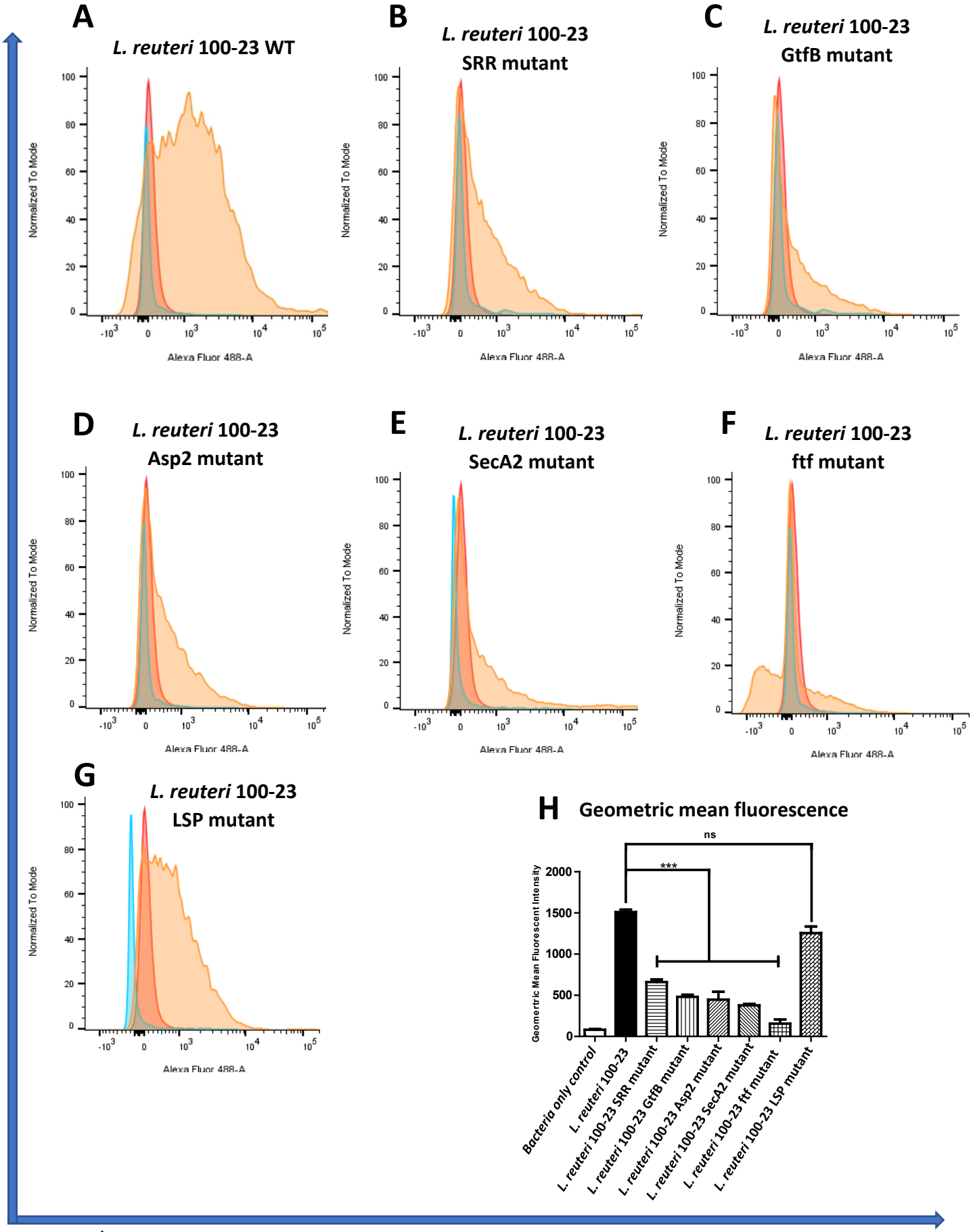
A
L. reuteri 100-23 WT
B
L. reuteri 100-23
SRR mutant
C
L. reuteri 100-23
GtfB mutant
D
L. reuteri 100-23
Asp2 mutant
E
L. reuteri 100-23
SecA2 mutant
F
L. reuteri 100-23
ftf mutant
G
L. reuteri 100-23
LSP mutant
Normalized To Mode
Alexa Fluor 488-A
H
Geometric mean fluorescence
Geomertric Mean Fluorescent Intensity
ns
***
Bacteria only control
L. reuteri 100-23
L. reuteri 100-23 SRR mutant
L. reuteri 100-23 GtfB mutant
L. reuteri 100-23 Asp2 mutant
L. reuteri 100-23 SecA2 mutant
L. reuteri 100-23 ftf mutant
L. reuteri 100-23 LSP mutant
L. reuteri [%]
Binding to Dectin-2

Figure 57: Binding or *L. reuteri* 100-23 strains to recombinant hDectin-2.

A total of $2.5x10^7$ bacteria, grown in LDMII were incubated with purified Dectin-2. Following washing, the interaction to Dectin-2 was detected using primary anti-Dectin-2 antibody (1:1000). *L. reuteri* 100-23 **(A)** WT **(B)** *SRR* mutant **(C)** *GtfB* mutant **(D**) *Asp2* mutant **(E)** *SecA2* mutant and **(F)** ftf mutant **(G)** LSP mutant **(H)** summary. Fluorescence intensities were measured by FACS Calibur (BDBiosciences, USA). The data were analysed using the FlowJo software version 5.7.1 (Tree Star, Ashland, OR, USA).

Since Dectin-2 is a Ca^{2+} -dependent carbohydrate binding CTL, we examined the interaction safter addition of Ca^{2+} or ethylene glycol tetraacetic acid (EGTA), a calcium chelator (**Figure 57**). A higher binding between hDectin-2 and *L. reuteri* 100-23 WT and *LSP* mutant was observed in the presence of Ca^{2+} as compared to the bacterial only control (**Figure 57A and Figure 57G**). Furthermore, a significant reduction was observed between hDectin-2 and the *L. reuteri SRRP* and *ftf* mutants irrespective of the presence of Ca^{2+}. EGTA treatment (5 mM) abrogated binding of all *L. reuteri* 100-23 strains tested. These results suggest that putative ligands of Dectin-2 are expressed and accessible on *L. reuteri* 100-23 cell surface, and that both SRRP and EPS may play a role in may play a role in this interaction, further supporting the results of the Dectin-2 reporter cells (**Figure 54**).

7.3 Role of Dectin-2 in the interaction between *L. reuteri* 100-23 and BMDCs

To determine the role of *L. reuteri* cell-surface adhesins in the ability of the strains to induce Dectin-2-mediated immune response, we tested the effect of WT and mutant *L. reuteri* 100-23 strains on cytokine production by BMDCs derived from WT or Dectin-2 KO mice. Following treatment with *L. reuteri* strains, pro-inflammatory (TNF-α and IL-23) and anti-inflammatory cytokines (IL-10) concentrations were determined in supernatants of BMDC (WT or Dectin-2 KO) cultures by ELISA.

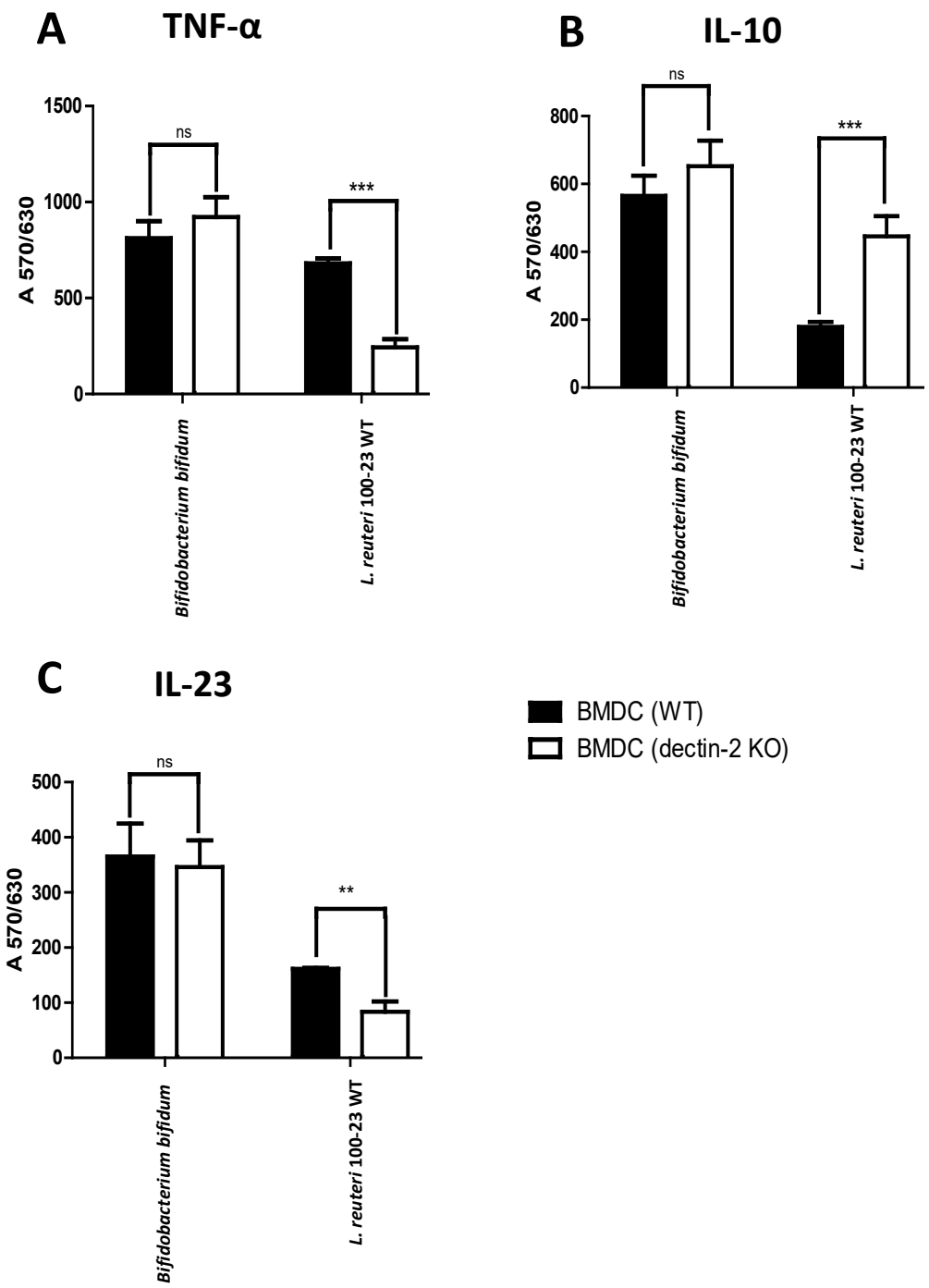

Figure 58: Effect of *L. reuteri* 100-23 strains on cytokine production by WT and Dectin-2 KO BMDCs.

Mouse BMDCs were co-incubated with *L. reuteri* 100-23 WT, or with *Bifidobacterium bifidum* as a control, for 18 h. The concentration of TNFα **(A)**, **(B)** IL-10 and IL-23 **(C)** cytokines was measured by ELISA.

Overall, treatment of the Dectin-2 KO BMDCs with *L. reuteri* 100-23 WT, resulted in a significant reduction ($p < 0.001$) of the proinflammatory cytokines TNF-α and IL-23 production as compared to the WT BMDCs (**Figure 58A** and **Figure 58C**), whereas the IL-10 production was significantly higher ($p < 0.001$) in the Dectin-2 KO BMDCs as compared to BMDCs from WT mice **(Figure 58B)**. In contrast, there was no differences in cytokine production between the WT BMDCs and Dectin-2 BMDCs following treatment with *B. bifidum* (used as a control), further supporting that the specificity of the interaction between Dectin-2 and the *L. reuteri* 100-23 strain.

7.4 Role of Dectin-2 in the internalisation of *L. reuteri* 100-23 by BMDCs

To further explore the potential role of Dectin-2 in *L. reuteri* DC-mediated host immune response, we investigated the ability of BMDCs isolated from Dectin-2 KO mice to recognise and phagocytose *L. reuteri* 100-23 strains using Imaging flow cytometry. Imagestream was used to quantify the internalisation rate of *L. reuteri* 100-23 WT and mutant strains into BMDCs. A control experiment was performed at 4°C. At this temperature, energy dependent uptake as well as passive diffusion are blocked due to the rigidity of the membrane that does not enable passive internalisation to take place (**Figure 59**).

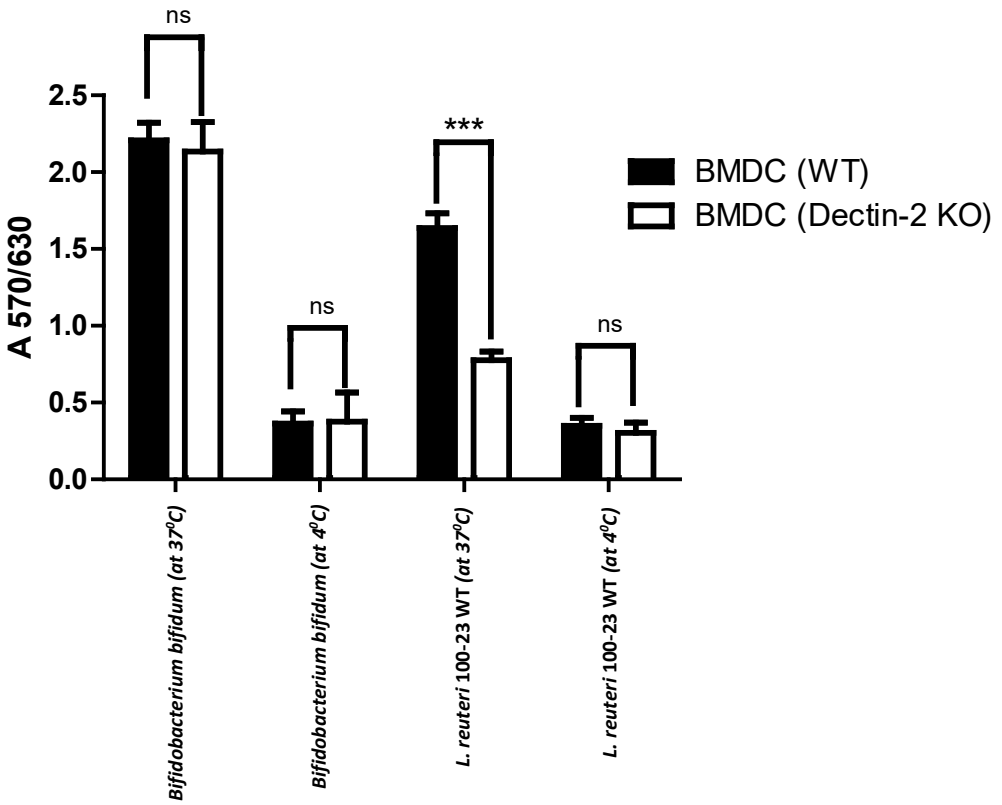

Figure 59: Effect of Dectin-2 deficiency on phagocytosis of *L. reuteri* 100-23 by BMDCs.

BMDCs from wild-type and Dectin-2 KO mice were differentiated in cell culture dishes at a density of 5.0×10^5 cells/mL in medium supplemented with GM-CSF for 7 days. BMDCs were then stained with Cell Trace Violet (CTV) for 15 miN at room temperature and stimulated with cFDA labelled *L. reuteri* strains. Coculture of CTV stained BMDCs and cFDA labelled bacteria was then performed at 37°C for 1 h. Internalisation score calculated by Amnis IDEAS software: distribution of IS of at least 10,000 cells treated for 4 h at 37 °C, and corresponding mean value of IS ± SD of 3 independent experiments.

The internalisation of *L. reuteri* 100-23 WT strain was 2-fold lower in the BMDCs harvested from Dectin-2 KO mice as compared to the BMDCs from WT mice (**Figure 59**). Although the BMDCs were able to internalise *B. bifidum* used as a control, there was no difference in internalisation rate between BMDCs from WT and Dectin-2 KO mice. In addition, the internalisation of *L. reuteri* 100-23 WT was significantly reduced at 4°C as compared to rate obtained at 37°C. These results indicate that Dectin-2 contributes to mediating the uptake and engulfment of *L. reuteri* 100-23 by BMDCs.

7.4 Discussion

In this chapter, we investigated the role of C-type lectins in the DC-mediated immune response to *L. reuteri* 100-23 (rodent isolate) *in vitro*. Using reporter cells specific for individual CTLs, we showed that *L. reuteri* 100-23 was recognised by mDectin-2 and SIGN-R1 but not by mDectin-1. The interaction to Dectin-2 and SIGNR1 appeared to be glycan-mediated as mutation of the CRD domain abrogated the interaction between the CTLs and *L. reuteri* 100-23.

Dectin-2 and SIGNR1 are important CTLs involved in the recognition of several pathogens including *C. albicans* and *Aspergillus umigatus*, driving the production of pro-inflammatory cytokines and inducing protective immunity in the host[649, 650]. The CRD of Dectin-2 exhibits specificity toward high mannose glycoconjugates mainly found in fungi[243]. Similarly, determination of the crystal structure of DC-SIGN and SIGNR1 has revealed high-affinity binding to an internal feature of high-mannose oligosaccharides found in fungi[651]. However, comparison of the lectin activity of SIGNR1 with Dectin-2 showed that they exhibited distinct binding profiles[243]. Glycan array analysis of the specificity of the CRD of Dectin-2 indicated that Dectin-2 was very specific for high-mannose structures with high recognition of $Man_9GlcNAc_2$>$Man_8GlcNAc_2$ and also some recognition of $Man_7GlcNAc_2$[243], whereas SIGNR1 demonstrated specificity for mannose-, fucose- and GlcNAc-terminating oligosaccharides[652]. Here, we showed that SRRP contributed to the interaction between *L. reuteri* 100-23 and mDectin-2 and SIGNR1. The direct interaction of SRRP with recombinant hDectin-2 was confirmed by flow cytometry. The overall amino acid sequence of hDectin-2 has been shown to display 66.5% identity with that of mDectin-2 and shares many common features with mDectin-2, including the EPN motif in the CRD, a short intracellular tail with no tyrosine residues and identical domain structures with the same numbers of amino acids in each domain[653, 654], which is consistent with the capacity of both mDectin-2 and hDectin-2 to recognise $SRRP_{100\text{-}23}$.

Using GC-MS analysis (**Chapter 5**), we showed the presence of Gal, as well as smaller amounts of Glc, GalNAc, GlcNAc and Rha in $SRRP_{100\text{-}23}$. It is possible that, although primarily shown to recognise mannose, Dectin-2 and SIGNR1 may also be able to recognise the GlcNAc residues that decorate the $SRRP_{100\text{-}23}$ since the adhesin does not contain mannose residues. Both lectins are expressed in myeloid cells such as DCs which are professional APCs pivotal in the instruction of T cell responses[598]. The functional differences between these receptors, therefore, will depend not only on their relative affinities for ligand but also the post-ligation events such as intracellular trafficking and signal transduction.

DC-SIGN (SIGN-R1 human homologue) has previously been involved in the interaction between cell surface proteins of commensal strains and C-type lectins. A recent study showed that the commensal *Propionibacterium* UF1 surface glycosylation of the large surface layer protein A directs the intestinal homeostasis via SIGNR1 and protects the host against proinflammatory signals inducing colonic tissue damage in mice[655]. In addition, *L. acidophilus* surface layer protein A has been shown to recognise DC-SIGN and modulate the production of IL-10 and IL-12 by murine DCs *in vitro*[656]. In another study, the surface layer proteins of *L. acidophilus* was demonstrated to block Chikungunya virus and Semliki Forest virus infection via DC-SIGN *in vitro*[657]. In contrast, most of the studies describing ligands for Dectin-2 have focused on their capacity to bind pathogen-derived carbohydrate ligands. This unique interaction between Dectin-2 and *L. reuteri* 100-23 (which is considered autochthonous in mice) may suggest immunomodulatory role in gut homeostasis.

Dectin-1 recognises β-glucan which are carbohydrates widely expressed on the cell wall of many fungal organisms such as *Pneumocystis carinii* or *C. albicans or Aspergillus fumigatus*[220]. Despite the presence of Glc residues on the SRRP, mDectin-1 was not able to interact with *L. reuteri* strains. Using glycan microarrays, mDectin-1 has been shown to exclusively recognise β-1,3 and β-1,6 linked glucans (but not those containing solely β-1,6 linkages) from a variety of sources, including yeast, other fungi, plants, and bacteria[239, 658]. In these studies, the minimum length required for detectable binding is a 10- or 11-mer, indicating the specificity of the binding. In addition to the backbone chain-length of β(1,6)-branching has been shown to affect binding to Dectin-1[647]. To gain structural insights into the lack of interaction between Dectin-1 and *L. reuteri*, linkage analysis should be performed on the surface proteins found on the *L. reuteri* 100-23 strains.

Our results also indicate that EPS plays a role in the interaction of the bacteria to mDectin-2 and purified hDectin-2 as *L. reuteri* EPS mutant showed a reduced interaction to mDectin-2 cell reporter cells and

purified hDectin2. Total sugar analysis of polysaccharide extracted from sucrose-containing liquid cultures of strain 100-23 extracted showed that this EPS was a levan (β-2, 6-linked fructan)[387]. It may be that Dectin-2 could recognise fructose which is the main sugar found (98.7% w/w) on *L. reuteri* 100-23 EPS[387]. Levan produced by *L. reuteri* 100-23 has been shown to increase proportions of regulatory T cells Foxp3+ in the spleen of mice[387] and it is therefore possible that *L. reuteri* 100-23 EPS-Dectin-2 interaction may be involved the induction of Treg cells to generate immunological tolerance towards these commensal bacteria in the murine gut. Further work involving coculture of *L.* reuteri 100-23 WT or *EPS* mutant bacteria-primed WT or Dectin-2 KO DCs with T cells will need to be performed to validate this hypothesis.

Using BMDCs lacking surface expression of Dectin-2, we also showed that Dectin-2 contributed to the *L. reuteri* 100-23–induced production of cytokines, as the absence of Dectin-2 led to a reduction in proinflammatory cytokines TNF-α and IL-23 and an increase in IL-10 production as compared to WT BMDCs. IL-23 and TNF-α have been implicated in several autoimmune inflammatory disorders such as colitis, gastritis, psoriasis and arthritis[659, 660-662]. The downregulation of proinflammatory cytokines by *L. reuteri* 100-23 in absence of Dectin-2 is concurrent with the reported activator properties of Dectin-2. Indeed, Dectin-2-mediated recognition of *C. albicans* leads to induction of IL-1β and IL-23 production by macrophages[660]. In addition, Dectin-2 KO mice have been shown to be more susceptible to *Candida glabrata* infections, showing a defective fungal clearance[244]. The increased susceptibility to infection was accompanied by lower production of Th1 and Th17-derived cytokines by splenocytes of Dectin-2 KO mice. However, it is worth noting that*, in vivo*, Dectin-2 has also been implicated in protecting the host by dampening the excessive inflammatory responses through the expression of putative Dectin-2 ligands on regulatory T cells leading to blockage of Dectin-2-mediated signalling and affected the immune tolerance [254]. Another study showed that *in vitro* Dectin-2 is essential for the production of anti-inflammatory cytokine, IL-10 in mouse BMDCs treated with mannose-capped lipoarabinomannan, which is a major lipoglycan of *Mycobacterium tuberculosis*[663]. In the future, we will investigate the downstream consequences of Dectin-2-ligand interaction using Dectin-2 deficient mice *in vivo*.

In addition, the internalisation of *L. reuteri* 100-23 by BMDCs was abolished in BMDCs lacking Dectin-2 while engulfment of *B. bifidum* was not affected, suggesting that Dectin-2 is involved in *L. reuteri* 100-23 internalisation by DCs, possibly through its interaction with $SRRP_{100\text{-}23}$ or EPS. Recently, deficiency in Dectin-2 has been shown to significantly impair the uptake and phagocytosis of several fungal organisms such as *Candida*, *Saccharomyces*, *Malassezia*, and *Mucor* species[664]. Dectin-2 has previously been shown

to employ Fc receptor γ chain (FcRγ) signalling to induce internalisation, activate NF-κB, and up-regulate production of TNF-α[665]**.** In chapter 6, we showed that $SRRP_{100-23}$ is a crucial *L. reuteri* 100-23 surface component in the activation of the NF-κB-mediated cascade. Moreover**,** In the previous chapter, we also demonstrated the importance of $SRRP_{100-23}$ in the internalisation of *L. reuteri* 100-23 by BMDCs. These results suggest that the phagocytosis and enhanced level of inflammatory immune responses provoked by *L. reuteri* 100-23 *in vitro* is dependent on the interaction between $SRRP_{100-23}$ and the surface Dectin-2 receptors of BMDCs. Therefore, we can speculate that recognition of GlcNAc residues that decorate $SRRP_{100-23}$ by Dectin-2 on BMDCs may contribute to the initiation and modulation of both innate and humoral immunity.

Taken together, these data provide new insights into the mechanisms by which *L. reuteri* strains exert immunomodulatory properties via the interaction of *L. reuteri* host-specific adhesins with C-type lectins expressed on DCs.

CHAPTER 8: GENERAL DISCUSSION AND FUTURE PERSPECTIVES

It is now well established that the gut microbiota is essential for human health and that alterations in its functional composition, can lead to disease[666]. Large-scale research programs such as the pioneer projects, Meta Hit[667] or Human Microbiome Project[668], and more recently Earth Microbiome Project[669], have allowed the identification of new strains and/or new microbial functions and components supporting the development of potential prophylactic or therapeutic applications[670]. One approach is the use of live biotherapeutics, which are defined as products containing a live microorganism and is applicable to the prevention, treatment, or cure of human diseases[671, 672]. There are some prerequisites for becoming potential live biotherapeutic product: to survive in low pH and enzyme-enriched environments, to adhere to epithelium for host-bacteria interaction, competition with pathogenic microorganisms, and most importantly, safety[672]. Another approach is the use of molecules derived from gut bacteria, microbiome-derived biologics, such as peptides or proteins being able to recapitulate the function of the bacteria[673], or mimic the effect of human hormones[674, 675]. The full exploitation of these strategies to influence the microbiota for the benefit of human health, however, requires a better understanding of this microbiome–host relationship. *Lactobacillus* species are one of the most widely used live biotherapeutic products and can be found in a large variety of food products throughout the world[676].

L. reuteri is an excellent model organism to identify host-specific immunomodulatory properties of commensal bacteria because it is widespread gut symbiont found in many vertebrate hosts and one of the first to colonise the human GI tract[386]. The underlying mechanisms by which *L. reuteri* exerts its well-documented host-specific therapeutic effects in the gut are not fully characterised[677]. At the molecular level, *L. reuteri* adhesion to host tissues in the GI tract has been shown to be mediated to a large degree by specific adhesins such as SRRPs expressed by strains isolated from rats and pigs[378, 678], MUB isolated from pigs[426, 485] and CmbA found on strains isolated from humans[432, 433]. Previous work in our group using a combination of bioinformatics analysis, lectin screening, LC-MS-based sugar nucleotide profiling, MALDI-ToF, and GC-MS analyses was used to show that *L. reuteri* can perform protein glycosylation[442] and that both MUB[511] and SRRP[442] are glycosylated. To date, the role of protein glycosylation systems in bacteria has been extensively studied in pathogens such as *Campylobacter jejuni*, *Streptococcus pyogenes* and *Burkholderia pseudomallei*, underscoring the importance of glycans in colonisation, immune evasion and survival of bacterial pathogens[679-685]. However, protein glycosylation systems are also found in commensal bacteria and therefore no longer exclusively associated with a canonical

virulence factor as defined by the criteria established by Falkow[533]. However, our current understanding of the nature and function of protein glycosylation in commensal bacteria is far from complete[534]. In this study, we investigated whether differences in protein glycosylation of *L. reuteri* adhesins, may play a part in the host specificity, but also the immunomodulatory properties of *L. reuteri* strains by interacting with human lectins. Using GC-MS and lectin analysis, we confirmed that *L. reuteri* 100-23 and ATCC 53608 strains can perform protein *O*-glycosylation and showed that $SRRP_{100\text{-}23}$ and MUB_{53608} are modified with Hex-Glc-GlcNAc and di-GlcNAc moieties, respectively.

Among all the *L. reuteri* strains tested, the pig isolate (ATCC 53608) showed the highest binding to mucins and that the interaction was shown to be dependent on the surface expression of MUB. The importance of MUB in autoaggregation and interaction of the bacteria with mucin was further confirmed under physiologically relevant shear conditions. The binding to mucin is consistent with MUB demonstrated ability to bind to mucus[490] therefore contributing to the retention of the isolate *L. reuteri* ATCC 53608 bacteria within the outer mucus layer. The role of MUB does not seem to be restricted to mucus binding. Here, surface expression of MUB was also implicated in the interaction with epithelial cells, further highlighting the importance of MUB in the interaction between *L. reuteri* ATCC 5308 and the host. The apparent mucin and epithelial cells-binding properties reported here for strain ATCC 53608 could reflect both the mucus recognition abilities and the propensity of this strain to auto aggregate, as previously reported[388]. Using the same approach, we showed that CmbA was implicated in the interaction between human-derived epithelial cells and the human isolate ATCC PTA 6475 strain. The ability of ATCC 53608 and ATCC PTA 6475 to bind to various intestinal surfaces may be used as a strategy to reduce infection at the mucosal surface, as demonstrated using *in vitro* organ culture from human biospies[432]. Geared for digestion and absorption, some sites of the GI tract present harsh conditions for microorganism colonisation[386], with for example low pH conditions caused by gastric acids and bile salts in upper small intestine[386, 686]. Here, we demonstrated that the adhesion ability of *L. reuteri* ATCC 53608 strain to mucin was not affected by low pH conditions caused by gastric acids. This intrinsic tolerance to GI stress factors is a key element in guaranteeing the performance bacterial strains used in live biotherapeutic products since a large number of viable microorganisms must reach the intestine in order to produce a beneficial effect on health[501].

In contrast to ATCC 53608 and PTA 64715, we found that the rodent isolate *L. reuteri* 100-23 strain was not able to bind to mucins nor to the human derived epithelial cells. Therefore, an *ex-vivo* adhesion assay based on tissue harvested from mouse forestomach was employed to investigate the role of

glycosylated SRRP in the interaction with *L. reuteri* 100-23. Our results clearly demonstrated both the expression and glycosylation of SRRP is important for the adherence of the bacteria to the mice forestomach. This is line with the ecological niche of *L reuteri* in rodents which is known to colonise the forestomach epithelium of mice and previous reports showing that mutant strains deficient in SRRP and/or its dedicated transport system (the SecA2-SecY2 pathway) completely abrogated biofilm formation[378]. The EPS from the rodent isolate was also implicated in the interaction with the cornified, stratified squamous epithelium which line the proximal area of mice forestomach, supporting previous studies showing EPS biosynthesis play an important role in *in vitro* biofilm formation of *L. reuteri* 100-23[387]. Together, these results indicate that initial adhesion facilitated here by SRRP and EPS may represent the most significant step in biofilm formation in mice, likely conferring host specificity[378]. Such interactions with the host may also be mediated by lectins found in the GI tract.

Mammalian lectins fulfil several physiological functions, such as adhesion to other cells, endocytosis, and when expressed on immune cells can also mediate immune modulation through their intracellular signalling[687]. Host lectins are mainly expressed by immune cells, but some are also produced by epithelial cells[518, 519]. They can be secreted or bound to the cell membrane (transmembrane proteins). Here, we tested the ability of *L. reuteri* glycosylated adhesins to bind to host lectins in the gut. We showed that MUB could bind to the β-galactoside-binding lectin, Gal-3 and D- galactofuranose-specific lectin, hINTL-1, consistent with the glycosylation of the adhesins with galactose residues. D-galactofuranose is often found in fungal cell walls and glycoproteins[529] as well as in carbohydrate structures from important human parasites and bacterial pathogens[530]. However, its presence on commensal bacteria remains to be determined. A recent study investigated the binding of hINTL-1 to saccharide residues that possess a terminal 1,2-diol, sugars present in bacterial LPS, showing that hINTL-1 avoids binding prevalent glycans with a terminal 1,2-diol[688] which suggests that the lectin has evolved to recognize distinct bacterial species. Gal-3, on the other hand, exhibits pleiotropic biological functions such as influencing cell growth, cell adhesion, cell-cell interaction, and as a pre-mRNA splicing factor[159]. Within the intestinal tract, Gal-3 is detected predominantly in the villus tips[520] and in the mucus layer[521] and interacts with MUC2, the main secreted mucin in intestinal mucus[522, 523]. Extracellular Gal-3 can act as a PRR and interact with a range of pathogenic bacteria including *Neisseria gonorrhoeae*[524], *C. albicans*[525], *Schistosoma mansoni*[526] *and Trypanosoma cruzi*[527, 528], further regulating the course of an infection by virtue of its effects on the cells of the innate immune system such as neutrophils, monocytes, and dendritic cells[689]. In addition, one study reported the interaction between Gal-3 and

commensal bacteria, showing two strains of *Bifidobacterium longum* subsp. *infantis* interacted with Gal-3 to a greater extent than did the pathogenic bacteria, *Escherichia coli* NCTC 12900[171].

Previous *in vitro*, cell-based, and force spectroscopy assays in our lab has also demonstrated that mucins could directly interact with Gal-3[544]. The glycan-lectin interaction between galactose moieties on the MUB and Gal-3 observed here may define how *L. reuteri* ATCC 53608 colonise the host epithelial cells and mucus layer of the GI tract. Indeed, Gal-3 is a major component of intestinal mucus layer, as shown by proteomics of mucus in GI tract of mice[545]. The ability of *L. reuteri* ATCC 53608 strain to interact with these intestinal lectins *in vitro* may suggest an additional role for MUB in mediating *L. reuteri* spatial colonisation. MUB-mediated binding to mucins, Gal-3 or hINTL-1 may ensure that *L. reuteri* remains confined to the outer mucus layer [546] although *in vivo* work is required to fully characterise the interaction between Gal-3 and hINTL-1 and *L. reuteri* in the GI tract.

Arguably the most intriguing feature of *L. reuteri* and a likely underpinning of its health-promoting effect is the ability to modulate the host's immune system. Empirical evidence for an immunoregulatory effect was achieved in several experimental models of colitis, where *L. reuteri* was highly efficient in reducing inflammation[453, 690-693]. It is also clear that the immunomodulatory effects of *L. reuteri* strains are strain-dependent, exerting different DC activation patterns *in vitro*[268, 576, 577]. Although not fully understood, it is likely that the sum of bacterial cell surface-derived and soluble factors and/or EPS contributes to the development of different immune responses induced by *L. reuteri* strains. Here, we demonstrated that host strain-specific glycosylated adhesins $SRRP_{100\text{-}23}$ and MUB_{53608} contribute to the immunomodulatory effects of *L. reuteri* 100-23 and ATCC 53608 *in vitro* by (i) mediating enhanced surface activation marker expression and (ii) inducing pro-inflammatory and anti-inflammatory cytokines by DCs. Glycosylation of lactobacilli cell-surface proteins such as SRRP may yet be another mechanism contributing to the overall health benefit of commensals on the host immune system to establish homeostasis in the gut[575, 694]. CTLs are a class of PRRs that are expressed by a broad spectrum of cells and are involved in the induction of pathogen-specific gene expression profiles, driving both innate and adaptive immunity[213]. The mechanisms by which *L. reuteri* adhesins exerts immunomodulatory effects was further investigated using purified and cell reporter assays specific for different CTLs. From their first discovery, CTLs have been studied mainly for their involvement in anti-fungal and anti-viral immunity, with research on CTL-bacterial interactions lagging[645]. Here we showed that showing that *L. reuteri* 100-23 was recognised by hDectin-2, mDectin-2 and SIGNR1 and that the CRD domain of both mDectin-2 and SIGN-R1 were required for the interaction to occur between the bacteria and the CTLs, indicating the specificity of the

protein-glycan interaction. In addition, we demonstrated that $SRRP_{100-23}$ mediated the interaction between the bacteria and Dectin-2 and SIGNR1. So far, ligands recognised by Dectin-2 are mainly high mannose residues that are present on fungal cell surfaces[243, 664, 695-697] and therefore the interaction with $SRRP_{100-23}$ is unexpected. Although one study has shown that commensal yeasts such as *Saccharomyces cerevisiae* and *Kazachstania unispora* can induce immunomodulatory functions via Dectin-2 in the mice[698]. Therefore, we can speculate that recognition of the $SRRP_{100-23}$ by Dectin-2 on DCs may contribute to the initiation and modulation of anti-fungal immunity. We showed that Dectin-2 contributes to the *L. reuteri* 100-23-induced production of cytokines, leading to a decrease in proinflammatory cytokines and an increase in anti-inflammatory cytokine in DCs lacking Dectin-2. In addition, we showed that the internalisation of *L. reuteri* 100-23 by DCs was dependent on the surface expression of Dectin-2 on DCs, suggesting that the phagocytosis and enhanced level of inflammatory immune responses provoked by *L. reuteri* 100-23 *in vitro* is dependent on the interaction between $SRRP_{100-23}$ and the surface Dectin-2 receptors of BMDCs.

The discovery that *L. reuteri* interact with Dectin-2 sheds new light on the immunomodulatory mechanisms of *L. reuteri* strains and may be relevant to the translation of therapeutic applications. With most of the receptors in the Dectin-2 ligands yet to be fully characterised[245], these first glimpses of the diverse repertoire of bacterial ligands and functions reveals an exciting area of future research. Work is currently on-going in the Lab to validate the findings *in vivo* using Dectin-2 k/O mice treated with *L. reuteri* 100-23 strains deficient in SRRP and/or its dedicated glycosylation and transport system (the SecA2-SecY2 pathway).

Overall, our findings indicate dual function of glycosylated cell surface proteins, such as MUB and SRRP in mediating bacterial adhesion to mucins and epithelial cells as well as immunomodulation.

This work also confirmed the immunomodulatory properties of *L. reuteri* 100-23 EPS. EPS production by *L. reuteri* 100-23 may represent a function acquired by the bacterial species for life in GI environments[387, 699] that has subsequently been diverted to novel uses, including immunomodulation, that aid in colonisation of the murine gut. Structural and immunomodulatory differences have been shown among EPS of *L. reuteri* isolated from intestines of mice with experimentally induced IBD compared to those of healthy mice[405] which suggests that, upon gut inflammation, *L. reuteri* strains switch to producing EPS with specific motifs that are absent from *L. reuteri* strains found in a healthy gut. Here we showed that *L. reuteri* EPS structure was conserved between *L. reuteri* 100-23 wildtype and Asp2 mutant strains, with the main structure being levan (β-2, 6-linked fructan), consistent with

previous work[387]. It is likely that Dectin-2 may also recognise fructose which is the main sugar found (98.7% w/w) on *L. reuteri* 100-23 EPS[387].

We further revealed that BEVs secreted by *L. reuteri* 100-23 and ATCC 53608 could mediate bacteria-host interactions. Much of the knowledge of the role of BEVs in mediating the host's immunity is derived from studies of pathogenic bacterial outer membrane vesicles, showing their contribution to pathogenesis via delivering virulence factors to target cells[572, 573]. Recently, however, studies have revealed that BEVs derived from several probiotics and commensal bacteria, such as *Bacteroides fragilis*[615], *Akkermansia muciniphila*[616], *Escherichia coli* Nissle 1917[617], *Bifidobacterium longum*[618] and *L. paracasei*[619], have been shown to exert similar pro-inflammatory and anti-inflammatory effects. Consistent with these studies, we showed that *L. reuteri* 100-23 and ATCC 53608 produced relatively high quantities of BEVs *in vitro,* and treatment of BMDCs reproduced the immunomodulatory effects as their parental bacteria. Due to the unique nano-scale structure of the lipid membrane-encapsulated vesicles, BEVs have been shown to drive the long-distance transport of interior molecules throughout the intracellular compartments in a concentrated, protected and targeted manner[620]. These effects have been shown to be mediated by vesicular proteins. Here we hypothesised that BEVs may be harbouring the glycosylated adhesins, and therefore mediating their interaction with lectins expressed immune cells.

Our data show that BEVs are derived from the bacterial plasma membrane. Comparative proteomic analysis revealed that most of the BEVs proteins were predicted as cytoplasmic. Previous proteomic analyses of BEVs from other *L. reuteri* strains species have also revealed a predominance of cytoplasmic proteins. These findings are in accordance with the previously described composition of BEVs derived from other *L. reuteri* strains[409]. This is not surprising, as there is extensive literature reporting immunogenic and protective capacities of bacterial cytoplasmic proteins[700-706]. According to the distribution of biological functions analysed, most of these proteins were classified into proteases, metabolic enzymes, nucleic-binding proteins, transporter and membrane proteins and structural components of the ribosome, suggesting that *L. reuteri*-derived BEVs may also be involved in metabolism, transporter activity, translation and transcription, signalling and stress, etc.

It is conceivable that *L. reuteri*-derived BEVs could modulate the functions of the host's immune cells *in vivo* that are inaccessible to the whole bacterium in a healthy state due to spatial segregation. Thanks to their small size and circular shape, BEVs have been shown to go through the mucosal surface. For example, BEVs released by *Staphylococcus aureus*[707] and *Streptococcus pneumoniae*[708] have been shown

to fuse in a cholesterol-dependent manner with the plasma membrane of human epithelial cells. Moreover, BEVs from *L. rhamnosus*[709] and *Bifidobacterium longum*[618] have been shown to migrate to other tissues and cells to modulate the host immune system via pass through the intestinal mucus layer. BEVs could be used as therapeutic strategies to convey anti-inflammatory effects to the gut. Such strategies have been used to deliver therapeutics compounds such as for various payloads, including DNA, small interfering RNAs, peptides or proteins, and chemotherapeutic agents[710, 711]. In addition, previous studies have shown that vesicle vaccines can elicit a strong, lasting immune response in animal models[712]; however, these studies rely on artificially created vesicles that may not be effective in presenting the most efficacious antigens[620]. In the future, *L. reuteri*-derived BEVs could be designed for the development of novel vaccine candidates and adjuvants.

In conclusion, we have shown that *L. reuteri* cell surface glycosylation plays a crucial role in the interaction with the host's immune system. While cell surface polysaccharides have been extensively studied, it is important to consider the role of glycans found on adhesins in the interaction of commensal bacteria with the host to better define strain-specific components influencing host health and prevention or amelioration of disease.

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